University of Alberta

Alteration of the Protein Fraction of Bovine Milk

by

William Rattray ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Alteration of the Protein Fraction of Bovine Milk** submitted by **William Rattray** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Food Science.**

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Abstract

Protein standardization of milk, which is of considerable interest to the International Dairy Federation, refers to small adjustments of the protein content, within the limits of natural variability. In this study, the feasibility of adding different ultrafiltration (UF) permeates to milk to standardize the protein content 'down', from ~ 3.4% to the range ~ 2.4 - 3.2% protein, was investigated, in terms of the impact of UF permeate on the nutritional, sensory quality and heat stability of milk. When pasteurized milk was 'down'-standardized with skim milk UF permeate, changes in nutritional or sensory quality were small and high heat stability was preserved. The suitability of skim milk UF permeate was confirmed by the manufacture of protein 'down'-standardized, ultra-high temperature (UHT) milk; such milk exhibited acceptable sensory quality and excellent stability during UHT heating and subsequent storage for up to 12 weeks. Permeate made by the UF of quarg acid whey was unacceptable for protein down standardization of UHT milk; its presence led to off-flavours, a reduced heat stability of milk and high sedimentation during storage.

Modification of the protein fraction of milk, by enrichment with α-lactalbumin or β-lactoglobulin, to increase the ratio of whey protein to casein, without altering the total protein content, led to a general decline in heat stability at 140°C, over the pH range 6.4 - 7.1. The negative impact of β-lactoglobulin on heat stability was much greater than that of α-lactalbumin.

Upon acidification of milk to pH 4.8, the freezing point (FP) declined, indicating dissolution of the colloidal salts of the casein. When acidified milk was neutralized to pH 10, the FP remained low, indicating that the acid dissolution of colloidal minerals was irreversible. The relationship between FP and the pH of milk was not altered by heat treatments as severe as 120°C for 20 min, followed by cooling to 20°C for 20 h, showing that no change in the milk salt equilibria occurred. The FP measurements showed that salting out of casein in milk, by NaCl, LiCl, $CaCl_2$ or Na_2SO_4, did not involve the binding of any of the added salts to protein.

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List of Abbreviations

[ ] = Concentration

aCa^{2+} = Calcium ion activity (mMolar)

aH^{+} = Hydrogen ion activity (mMolar)

aOH^{-} = Hydroxyl ion activity (mMolar)

α-F = Fraction of α-lactalbumin

α-La = α-Lactalbumin

AWP = Acid whey ultrafiltration permeate; whey derived from directly acidified skim milk

$B_{12}BP$ = Vitamin B_{12} binding protein

β-F = Fraction of β-lactoglobulin

β-lg = β-lactoglobulin

BSA = Bovine serum albumin

CCP = Colloidal calcium phosphate

CD = Circular dichroism

DNA = Deoxyribonucleic acid

DSC = Differential scanning calorimetry

ΔE^* = Activation energy (kJ mol^{-1})

ΔG_D = Free energy of denaturation (kJ mol^{-1})

ΔH_D = Enthalpy of denaturation (kJ mol^{-1})

ΔS_D = Entropy of denaturation (kJ mol^{-1} K^{-1})

$\Delta G^{\neq}$ = Free energy of activation (kJ mol^{-1})

$\Delta H^{\neq}$ = Enthalpy of activation (kJ mol^{-1})

$\Delta S^{\neq}$ = Entropy of activation (kJ mol^{-1} K^{-1})

EDTA = Ethylenediaminetetraacetic acid

EGTA = Ethylene glycol-bis (β-aminoethyl ether)-N, N-tetraacetic acid

FBP = Folate binding protein

FP = Freezing point (°C)

FTIR = Fourier transform infra red spectroscopy

FV = Furosine value (mg kg^{-1})

FWP = Acid whey ultrafiltration permeate; whey derived from fermented skim milk

G = Universal gas constant (8.31×10^{-3} kJ mol^{-1} K^{-1})

GT = Galactosyl transferase

HCT = Heat coagulation time (min)

HPLC = High pressure liquid chromatography

List of Abbreviations (-ctd-)

Ig = Immunoglobulin

IR = Infra red

k = Rate constant (s^{-1})

k_0 = Pre-exponential factor (s^{-1})

k_{sp} = Solubility product (dimensionless)

MCP = Micellar calcium phosphate

MR = Maillard reaction

MW = Molecular weight (1 Da = 1.6×10^{-24} g)

n = Reaction order (dimensionless)

ORD = Optical rotary dispersion

PAGE = Polyacrylamide gel electrophoresis

Q_{10} = Relative change in reaction rate caused by a change in temperature of 10°C

SDS = Sodium dodecyl sulphate

SMP = Skim milk ultrafiltration permeate

SMR = Skim milk ultrafiltration retentate

SWP = Sweet whey ultrafiltration permeate

P_D = Denatured protein

pH = - Log_{10} of proton activity (dimensionless)

P_N = Native protein

T = Temperature (°C)

T_D = Denaturation temperature (°C)

UC = Ultracentrifugal

UF = Ultrafiltration

WPC = Whey protein concentrate

Chapter I
Introduction

The protein fraction of bovine milk is probably the most intensively studied food protein system. Interest in the milk proteins is not only academic, but stems also from a desire to more fully exploit their unique functional and nutritional properties, in order to develop new dairy products and food ingredients; at present, protein is considered to be the most valuable constituent of bovine milk. The ability to manufacture a diverse range of dairy products from milk is due in no small part to the variety of ways in which the proteins can be manipulated: the manufacture of cheese or caseinates requires the enzymatic or pH-induced gelation of casein (Dalgleish, 1987; Mulvihill, 1992); the texture of yoghurt is due to the formation of a network between casein micelles and whey proteins, caused by the effect of heating and subsequent acidification on the milk proteins (Mulvihill and Grufferty, 1995); the ability to manufacture low-, medium- or high-heat milk powders depends on the influence of milk pre-heating on the proteins (Singh and Newstead, 1995).

The protein content of bovine milk displays a considerable degree of natural variability, depending on breed of cow, stage of lactation, nutritional status of the cow, climatic conditions, farming practices and other factors. For example, Canadian milk can contain from 2.75 - 4.09% (w/w) protein, and similar variability has been reported for a number of other countries (Higgins *et al.*, 1995). There is now a strong incentive to obviate this natural variability or to 'standardize' the protein content of milk. According to the International Dairy Federation, protein standardization, should involve only small changes in the protein content of milk, within the limits of natural variability, without alteration of the casein to whey protein ratio.

The most widely cited methods of protein standardization include the ultrafiltration (UF) of milk or the addition of a milk UF retentate to milk to increase or standardize 'upward' the protein content. In contrast, protein standardization 'downward' would involve he addition of milk UF permeate or lactose to milk. There is also the possibility of adding other UF permeate types to milk for protein 'down' standardization, such as permeate derived from the UF of acid or cheese whey. Protein standardization can be expected to cause only minor changes in the protein content of milk, but a number of benefits would result: increased nutritional uniformity of fluid milk, condensed milk and milk powder; more accurate valorization of milk and other dairy products by the

processors; better control of product behaviour during thermal processes such as evaporation; and convenient utilization of by-products of the dairy industry such as UF permeate or lactose.

It is desirable that protein-standardized milk resemble as closely as possible normal milk, especially if the milk is for direct consumption. Changes in nutritional value should be minimal, in order to maintain the image of milk as a high quality source of nutrients. Neither should the sensory quality be adversely altered; ideally protein-standardized and normal milk should be mutually indistinguishable, when they both have the same fat content. Protein-standardized milk should also exhibit a high heat stability, required to withstand industrial heat treatments. It would be desirable if protein standardization was not accompanied by changes in the freezing point (FP), because if the FP would be altered, then standardization might be equated with adulteration; measurement of FP is carried out routinely to detect the possible presence of extraneous water in milk.

More drastic alteration of the protein content of milk, with or without alteration of the casein to whey protein ratio, can be termed 'protein adjustment'. This can be achieved by the addition of milk protein concentrates, whey protein concentrates or whey protein fractions, in the dried or liquid state, to milk. Protein adjustment is already practiced to some extent by the dairy industry; protein-adjusted milk can be processed to obtain yoghurt with improved texture and stability or to enhance the yield of cheese (Puhan, 1992). Not much data are available on the physico-chemical properties of protein-adjusted milk. Information on the heat stability would be especially desirable, because inadequate heat stability would restrict the ability to process such milk. On the other hand, a reduced heat stability caused by protein-adjustment might be useful; de Wit (1989) obtained a number of texturized, cheese-like dairy products by heating skim milk which had been fortified with a whey protein concentrate.

Normal bovine milk contains ~ 160 mmoles of total salt L^{-1}, of which ~ 125 mmoles are truly soluble and ~ 35 mmoles are colloidal, the latter being associated with casein and to a lesser extent with some of the whey proteins (Holt, 1985). The freezing point (FP) of milk depends fundamentally on its molality (moles solute kg^{-1} solvent) which in turn is affected by the concentration of low molecular weight compounds, especially lactose and soluble salts. Colloidal milk salts exist as part of the casein micelle, a high molecular weight entity, and therefore do not contribute significantly

to the molality of milk. However, the milk salt system is dynamic; changes in temperature or pH alter the partitioning of milk salts between the colloidal and soluble states and hence the molality of milk. It follows that measurement of FP could be used to detect irreversible changes in the partitioning of milk salts. Measurement of FP could be used also to determine if the addition of salts to milk would result in their binding to proteins, which would contribute to an improved understanding of the mechanism by which added salts reduce the solubility of milk proteins.

In light of the importance, yet unavailability, of certain information on protein-standardized or protein-adjusted milk and on the influence of heating on the equilibria of milk salts, the general objectives of the present work were as follows:

- To ascertain the suitability of different UF permeate types (skim milk permeate, rennet casein whey permeate, acid casein whey permeate or quarg acid whey permeate) for protein standardization 'downward' (in the range ~ 2.4- 3.4%, w/w, protein) or milk UF retentate for standardization 'upward' (~ 3.4 - 4.0%, w/w, protein). The feasibility of these methods of protein standardization was investigated in terms of their impact on the heat stability, freezing point, nutritional value and sensory quality of milk, both on an experimental scale and on a pilot scale; pilot scale studies involved the manufacture of protein 'down'-standardized, direct or indirect ultra-high temperature (UHT) milk.

- To determine the heat stability of skim milk after protein-adjustment, where adjustment was carried out by enrichment of milk with industrially-produced whey protein preparations, namely, fractions of α-lactalbumin or β-lactoglobulin or a whey protein concentrate.

- To establish the influence of pH adjustment in combination with various heat treatments on the FP of milk and hence the extent of changes in milk salt equilibria. The effects of added salts on the FP of milk was also investigated to determine if added salts could bind to milk proteins and how this would relate to the reduction in protein solubility at increasing ionic strength.

As a prelude to a better understanding of the experimental results obtained in the present work, Chapter II is a review covering the fundamental structural properties of the major milk proteins, the nature of the milk salt system and its interactions with milk proteins, the response of milk proteins to heat, in model systems and in dairy products, and detailed information on the current *status quo* regarding standardization or adjustment of the protein content of milk. The experimental results are then presented in seven interrelated Chapters. In Chapters III - V, the impact of protein standardization on the heat stability, nutritional and sensory quality and freezing point of experimental milk samples is investigated. Chapters VI and VII extend the experimental work on protein standardization to a more practical situation - the pilot scale manufacture of direct or indirect UHT, protein-standardized milk. Chapter VIII discusses the impact of protein adjustment, by enrichment of milk with α-lactalbumin or β-lactoglobulin, on the heat stability of milk; Chapter IX investigates the use of freezing point measurements to assess changes in milk salt equilibria caused by pH adjustment, heat treatments or the presence of added salts. Finally, Chapter X summarizes the results of Chapters III - IX, concludes their relevance to the dairy industry, and outlines areas where future work would be desirable.

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Chapter II

Literature review

1 The milk protein system

Bovine milk contains ~ 30 - 35 g of protein L^{-1} and the protein fraction is very heterogeneous. The major milk proteins have been extensively characterized in terms of structural, physiological, genetic, nutritional, physico-chemical and functional properties and numerous reviews are available (Brunner, 1981; Fox and Mulvihill, 1982; Eigel *et al.*, 1984; Whitney, 1988; Fox, 1989; Kinsella and Whitehead, 1989; Swaisgood, 1982, 1992; Dalgleish, 1993; Martin and Grosclaude, 1993; Creamer and MacGibbon, 1996). On a mass basis, the bulk of the protein falls into one of two broad classes - the casein protein fraction and the whey or serum protein fraction (Figure II-1).

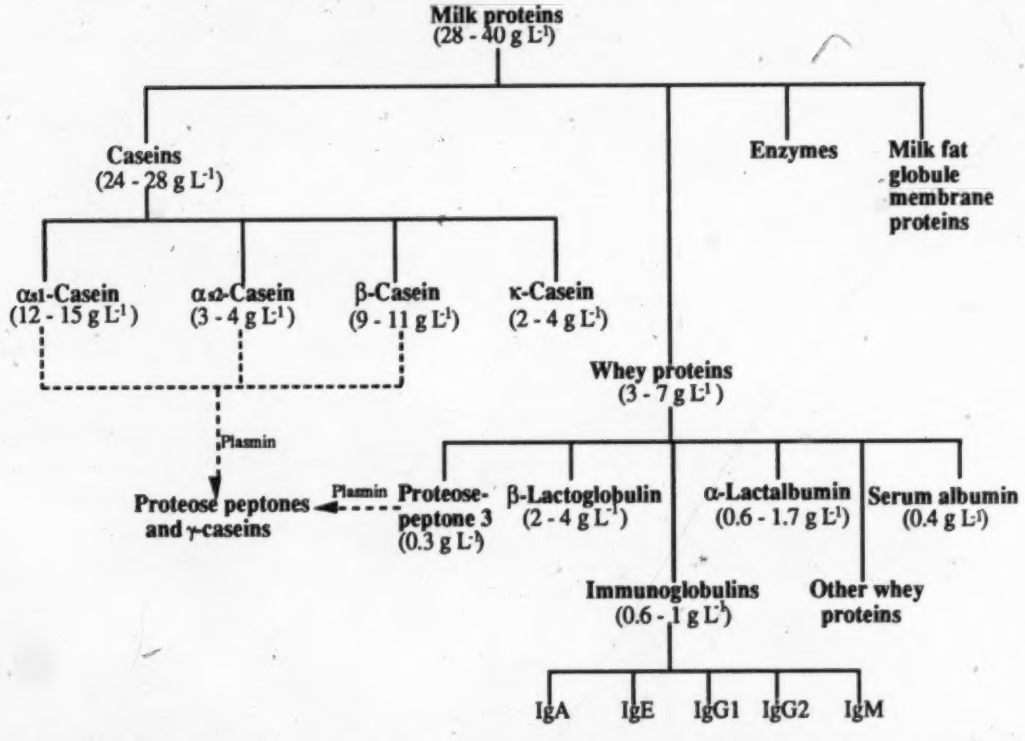

Figure II-1. Distribution of proteins in bovine milk. Broken lines indicate the hydrolytic activity of the indigenous milk enzyme plasmin to generate γ-caseins and proteose peptones.

The caseins, accounting for ~ 80% of total milk protein, consist of α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein. In milk, the four species of casein together with a large fraction of the mineral component are associated into roughly spherical aggregates, with typical diameters ~ 100 nm, termed casein micelles. The principal whey proteins are β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulin and proteose peptone, which make up ~ 20% of total milk protein and, in the normal milk environment, have a more limited tendency than the caseins to self-associate. Some fundamentally different structural and physico-chemical properties of the whey and casein proteins are shown in Table II-1.

Table II-1. Contrasting structural and physico-chemical properties of bovine whey and casein proteins.

Property	*Casein proteins*	*Whey proteins*
Structural		
Conformation	Extended[1]	Globular
Surface hydrophobicity	High	Low
Phosphorylation	High	Absent
Sulphydryl groups	Low	Low - high
Physico-chemical		
Sensitivity to pH	High	Low[2]
Sensitivity to ionic strength	High	Low[3]
Sensitivity to heat	Low	High
Sensitivity to centrifugal force	High	Low

[1]: Controversy exists as to the extent of tertiary structures in the caseins, but to date the most convincing evidence points to the absence of well-developed tertiary structures (see section 1.1).
[2]: The whey proteins are insensitive to pH in the sense that they are not acid-coaguable, but this does not rule out an effect of pH on their structures. For example, the quaternary structure of β-lg is sensitive to pH; α-la exhibits a pH-induced transition to the molten globule state (see section 1.2.1).
[3]: The whey proteins are relatively insensitive to salt because in milk they cannot be salted out. However salt ions can bind to some whey proteins to have a significant effect on structure and stability; for example, the binding of calcium to α-lactalbumin (see section 1.2.2)

As outlined by Fox (1989), the separation of whey proteins from casein proteins is readily achieved by four techniques, which reflect the fundamentally different structural and physico-chemical properties of the two protein fractions: (1) Adjustment of milk pH to 4.6, at 20°C, leads to precipitation of the caseins, while the whey or serum proteins remain

almost completely soluble; (2) Limited hydrolysis of κ-casein by chymosin, or other mildly proteolytic enzymes, causes the casein micelles to associate into a gel; when this gel is cut, syneresis of whey containing the whey proteins results; (3) Increasing the ionic strength of milk, by the addition of salt, reduces the solubility of casein, ultimately leading to its precipitation or 'salting out', while under the same conditions the whey proteins remain soluble; (4) Ultracentrifugation of milk causes the relatively large casein micelles to be sedimented and compacted into a pellet, while much greater centrifugal forces are required to sediment the whey proteins.

While the casein and whey protein fractions constitute the bulk of protein mass in milk, numerous kinds of milk protein occur at low or trace concentrations. These minor proteins include the indigenous milk enzymes and proteins which are an integral part of the milk-fat-globule membrane. The extensive knowledge of the milk enzymes was reviewed by Kitchen (1985) and Andrews *et al.* (1992), but information on the proteins of the milk fat globule membrane is more limited. Many of the enzymes cannot be classified as belonging to the whey or casein protein fraction, because some occur in both fractions and their distribution depends on the method of separation. Additionally, some enzymes are associated with the milk fat globule membrane. Therefore, it is convenient to assign the milk enzymes to a separate class. Some of the enzymes and minor whey proteins have known biological functions and/or are of technological significance, but most appear to be of little importance and may represent 'leakage' proteins transported passively from the blood during the biosynthesis of milk.

1.1 The casein proteins

The four major casein proteins of bovine milk are α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein, which represent ~ 45, 12, 34 and 10%, respectively, of total casein protein. Table II-2 shows some important structural, physico-chemical and hydrodynamic properties of the caseins. Distinctive characteristics of the caseins include, a high content of propyl and phosphoseryl residues, high hydrophobicities, relatively open structures and a tendency to undergo self-association. Within each species of casein, there is considerable heterogeneity, due to the existence of a number of alleles for each gene for casein synthesis, which give rise to genetic variants, and different extents of post-translational phosphorylation and, in the case of κ-casein, different extents of glycosylation.

Table II-2. Physico-chemical characteristics of isolated bovine casein proteins[1].

Protein	*Molecular weight (Da)*[2]	*Mean hydrophobicity (kJ residue^{-1})*[2]	*Isoionic pH*[2]	*Sulphydryl groups*	*Phosphoseryl groups*	*Propyl groups*	*Genetic variants*
α_{s1}-Casein	23 623	4.89	4.94	0	8 - 10	17	A B C D E
α_{s2}-Casein	25 238	4.64	5.37	2	10 - 13	10	A D
β-Casein	23 988	5.58	5.14	0	4 - 5	35	A1 A2 A3 B C D E
κ-Casein	19 006	5.12	5.90	4	1 - 2	20	A B

[1]: Adapted from Swaisgood (1992) where more detailed information is available.
[2]: Values are for the following casein species: α_{s1}-casein B-8P, α_{s2}-casein A-11P, β-casein A2-5P, and κ-casein B-1P.

The primary structures of all of the caseins have been established, on the basis of sequencing of the amino acid chain, the genomic DNA or complementary DNA, but reliable information on the secondary or tertiary structures has been much more difficult to obtain. A particular difficulty has been the inability to crystallize any type of casein, allowing for determination of three-dimensional structure by the classical technique of X-ray crystallography. A description of this method - determination of the structure of a molecule in its crystalline state from its X-ray diffraction pattern - is too complicated to go into here; however, Kitaigorodskii (1961) provides a detailed description of the theory of crystalline structure analysis. Methods used to determine the secondary structures of the caseins include experimental techniques such as optical rotary dispersive spectroscopy, circular dichroic spectroscopy or Fourier transform infrared spectroscopy (brief descriptions of these techniques are provided in section 3.3.4) and various theoretical techniques that attempt to predict secondary structures from the primary structure (Sawyer and Holt, 1993). The results of a number of studies using both experimental and theoretical methods were summarized by Swaisgood (1992) and Sawyer and Holt (1993) and it is clear that results of the various studies concur extremely poorly. For example, β-casein could contain 1 - 20 % α-helix, 0 - 33% β-structure and 15 - 31% turns (Swaisgood, 1992); similar discrepancies exist for information on the other caseins. Moreover, most of the techniques used to determine the secondary structure of the caseins have been applied to isolated casein monomers, but detailed information does not exist on the secondary and tertiary structures of the caseins when they are assembled into micelles.

The lack of precise spectroscopic data on the secondary structures of the caseins has contributed to controversy as to whether these proteins actually do or do not exhibit

substantial secondary and tertiary structures. Swaisgood (1992) opinionated that the caseins do show significant domains of local order; techniques, such as circular dichroism (Raap *et al.*, 1983; Creamer *et al.*, 1981), Raman spectroscopy (Byler *et al.*, 1988), Fourier transform infrared spectroscopy (Byler and Farrell, 1989) and thermodynamic calculations in conjunction with spectral data (Richardson *et al.*, 1992), revealed, despite their inconsistencies, that possibly 50% of the amino acid residues of α_{s1}-, α_{s2}- or β-casein could exist as α-helical, β-sheet or β-turn structures, while up to 75% of κ-casein could contain these structures.

Kumosinski *et al.* (1991a, b, 1993a, b), Farrell *et al.* (1993) and Richardson *et al.* (1992) attempted to predict the secondary and tertiary structures of the caseins using various sequence-based predictive techniques. The method behind their approach was inadequately described in their articles, but essentially involved the following: secondary structures were assigned to amino acid residues, when predicted by more than one technique or when strongly predicted by one technique, but not predicted against by another. Tertiary structures were then deduced by taking segments (80 - 100 amino acid residues) of polypeptide chain, with the predicted secondary structures, and calculating how these segments should be joined to one another, the assigned structures corresponding to states of low free energy. The results of this work were impressive, showing detailed three-dimensional structures for α_{s1}-casein, β-casein and κ-casein, but deserve to be treated with caution. The technique used was, in essence, an attempt to solve the 'protein-folding problem'; prediction of the tertiary structure of a protein from its known primary structure, on the assumption that the protein would spontaneously fold to acquire a state corresponding to a global or local minimization of total free energy; this topic was reviewed by Creighton (1990) and Baker and Agard (1994). As pointed out by Richardson *et al.* (1992), a particular difficulty with predictive techniques when applied to the caseins, was the high and relatively uniform distribution of propyl residues in the caseins; proline, being an imino acid induces sharp turns in the amino acid backbone which tend to disrupt, in a manner which is difficult to predict, the formation of α-helices and β-sheets. In some cases, the authors did not consider the effect of electrostatic[1] and hydrogen-bond

[1]: The term electrostatic refers to repulsive forces between unaccelerated charged groups. However, on a microscopic scale all molecules are in continuous motion both internally and in relative to one another. The acceleration of a charged particle generates an electromagnetic field and therefore strictly speaking the connotation 'electromagnetic forces' should be used. Nevertheless, in keeping with the widely accepted dogma, the term 'electrostatic forces', though technically incorrect, will be used throughout this treatise.

interactions on protein stability. Moreover, there is no example of a completely successful correlation between an experimentally and a theoretically determined protein structure.

Sawyer and Holt (1993) and Holt and Horne (1996) asserted that there was no convincing evidence for the existence of substantial secondary structures in the caseins. These authors questioned the reliability of optical rotary dispersive, circular dichroic and infra-red spectroscopy. These techniques are used normally to determine the secondary structures of a globular protein, using empirical rules generated from the spectra of another globular protein, with known secondary structures, as determined by X-ray crystallography; extension of these rules to the non-globular caseins could be misleading. Moreover the short time scales involved in these methods of analysis indicate that the secondary structures detected could be transient, rather than stable, features of the molecule. The authors favoured the use of ^{1}H-nuclear magnetic resonance spectroscopy for determination of secondary structures, and this technique indicated that the caseins display flexible, mostly random-coil structures. Nuclear magnetic resonance spectroscopy is based on the interaction of atomic nuclei with magnetic fields and the fundamentals of the theory were outlined by Hemminga (1992).

Aoki *et al.* (1991) suggested that the caseins should possess significant degrees of secondary structures in order for cross-linking to occur between casein submicelles (the building blocks of the casein micelle - see section 1.1.5). In the presence of 6 M urea, the binding of calcium, phosphorus and citrate to casein was inhibited, suggesting that disruptions of the secondary structures of the caseins by urea were responsible. However, the inhibition of mineral binding to casein caused by urea was limited; at 30 mM calcium (similar to the total concentration of calcium in milk), in the presence of 6 M urea, 41% of total calcium was bound, compared to 46% binding of calcium in the absence of urea. Kumosinski and Farrell (1993) observed that the infra-red spectra of isolated casein submicelles or submicelles which had aggregated in the presence of $CaCl_2$ into micelles, were almost identical, suggesting that if secondary structures existed they were unaltered by aggregation or, more likely, that the caseins did not possess secondary structures. Other evidence for the lack of secondary structures in the caseins is the fact that severe heating does not alter their infrared spectra (Parris and Purcell, 1990) or produce an endothermic heat response (Paulsson and Dejmek, 1990), implicating an absence of intramolecular hydrogen bonding.

Because of their high binding affinity for calcium and consequent ability to be precipitated by calcium, α_{s1}-casein, α_{s2}-casein and β-casein are referred to as the calcium-sensitive caseins, whereas κ-casein is relatively insensitive to calcium. In milk, the collective existence of the four types of caseins as micelles allows κ-casein to stabilize the calcium-sensitive caseins against precipitation at the relatively high calcium ion activity. Below, the properties of individual caseins are first described, to facilitate understanding of the subsequent description of the structure of the casein micelle. No information will be provided on the secondary and tertiary structures of the caseins, due to the extreme inconsistencies in the literature, and the controversy as to whether such structures do indeed exist.

1.1.1 α_{s1}-Casein

The protein α_{s1}-casein contains 199 amino acid residues and has a molecular weight (MW) of ~ 23 600 Da, the exact mass depending on the type of genetic variant and the degree of phosphorylation. Five genetic variants, A, B, C, D and E, of α_{s1}-casein have been identified in bovine milk, the B variant being predominant in the milk of western breeds of cattle. The primary structures of most of the genetic variants are known, determined chemically or deduced from the nucleotide sequence of the gene (Mercier *et al.*, 1971; Grosclaude *et al.*, 1973; Manson *et al.*, 1977; Swaisgood, 1992). Like the other caseins, most of the amino acid residues of α_{s1}-casein are hydrophobic, but 8 - 10 charged phosphoseryl residues occur. The hydrophobic and charged amino acid residues are not uniformly distributed throughout the molecule; three strongly hydrophobic domains, occurring at residues ~ 1 - 44, 90 - 113 and 132 - 199, have been identified, while residues ~ 41 - 80, which contain most of the phosphoseryl groups, form (at pH 6.6) a negatively charged cluster (Swaisgood, 1992). The presence of 25, approximately uniformly distributed, glutamyl residues in α_{s1}-casein, which carry a negative charge at the pH of milk, contribute also to the hydrophilicity of α_{s1}-casein.

1.1.2 α_{s2}-Casein

Bovine milk contains at least two genetic variants of α_{s2}-casein, termed A and D (Grosclaude *et al.*, 1979). Both variants contain 209 amino acid residues giving calculated MW's of ~ 25 300 Da. The primary structure of α_{s2}-casein has been determined both

chemically (Brignon *et al.*, 1977) and from sequencing of complementary DNA (Stewart *et al.*, 1987). This protein is the least hydrophobic of all the caseins, because it contains the greatest number (10 -13) of phosphoseryl residues and has also a relatively high (24) content of glutamyl groups. The charged phosphoseryl and glutamyl residues are distributed in three clusters, at amino acid positions 8 - 12, 56 - 63 and 129 - 133. The presence of these clusters is responsible for the extreme sensitivity, to pH and ionic strength, of the self-association reactions of α_{s2}-casein. Two cysteinyl residues are found on α_{s2}-casein; no conclusive information is available as to whether these residues are involved in the formation of disulphide bridges, which could be intra- or intermolecular, depending on whether the molecule was in the monomeric or polymeric state.

1.1.3 β-Casein

At least 7 genetic variants of β-casein have been identified in bovine milk, referred to as A^1, A^2, A^3, B, C, D and E, with the A^1 and A^2 variants being the most common in the milk of western breeds of cattle. The primary structure of at least the first five of these genetic variants is known (Jimenez-Flores *et al.*, 1987; Stewart *et al.*, 1987), each containing 209, mostly hydrophobic amino acid residues. The molecule contains a single polar domain with 4 - 5 negatively charged (at pH 6.6) phosphate groups, located near the N-terminus, followed by a long chain of mostly hydrophobic amino acid residues, making it strongly amphipathic. The self-association reactions of β-casein display a much greater sensitivity to temperature than the other caseins; cooling of milk to ~ 4°C leads to dissociation of β-casein from the casein micelle due to a weakening of intermolecular hydrophobic forces as the temperature is reduced (Creamer *et al.*, 1977; Law, 1996).

1.1.4 κ-Casein

From a number of viewpoints, κ-casein is disparate from the other three types of casein. The protein contains 169 amino acid residues, equivalent to a MW of ~ 19 000 Da, which is significantly less than the other caseins. It is the only casein that is glycosylated; it is the strongest candidate for the existence of significant degrees of secondary and tertiary structures; and it is relatively insensitive to precipitation by calcium, probably because the presence of a single phosphate group limits its ability to bind calcium. Two genetic variants of κ-casein, A and B, have been recognized in cow milk, both variants occurring

with almost equal frequency; their primary structures have been determined (Stewart *et al.*, 1984; Alexander *et al.*, 1988). The high amphipathicity of κ-casein is caused by the presence of a hydrophobic N-terminal domain (residues 1 - 105), while the remainder of the molecule is hydrophilic due primarily to the presence of a glycosyl group attached to threonyl residues. The glycosylated section of κ-casein contains three types of monosaccharide, namely, galactose, acetyl neuraminic acid and galactose-N- acetyl neuraminic acid (Doi *et al.*, 1979), which are covalently bound to threonyl residues in the form of a tri- or a tetrasaccaharide (Saito *et al.*, 1980). A κ-casein molecule can contain 1 - 4 tri- or tetrasaccharide carbohydrate moieties attached to threonyl residues at position 131, 133, 135, 136 or 142; there is also the possibility that ser_{141} is glycosylated (Zevaco and Ribadeau-Dumas, 1984). The four sulphydryl groups of κ-casein are probably involved in the formation of intermolecular disulphide bonds between κ-casein molecules when they are located in the casein micelle.

Cleavage of κ-casein by chymosin at phe_{105}-met_{106} splits the molecule into a glycomacropeptide, which contains the glycosyl groups and is therefore hydrophilic, and the remaining hydrophobic portion, termed para-κ-casein. This is the basis of cheese manufacture, in which the release of glycomacropeptide from the surface of casein micelles leads to a loss of the steric and electrostatic repulsive forces causing the micelles to aggregate into a network (Dalgleish, 1987; Green and Grandison, 1987). The phe_{105}-met_{106} bond is located in the vicinity of a proline-rich region of κ-casein and it is likely that the propyl residues increase the steric accessibility of this bond to chymosin. Richardson *et al.* (1992) proposed that when the phe_{105}-met_{106} bond was hydrolyzed, an α-helix (or possible β-sheet structure) located between pro_{101}-pro_{109} unraveled, making cleavage of this bond entropically favourable.

1.1.5 The casein micelle

In milk, all of the four types of casein exist in the form of relatively large, colloidal entities called casein micelles. Some properties of bovine casein micelles are shown in Table II-3. The casein micelles show a wide distribution of sizes, with diameters in the range of at least ~ 80 - 230 nm (McNeill and Donnelly, 1987) or possibly 20 - 600 nm (Schmidt *et al.*, 1973); on a mass basis ~ 80% of the casein in milk occurs in micelles of ~ 100 - 200 nm diameter (Lin *et al.*, 1971). The large size of the casein micelles enables them to scatter

visible light thus giving skim milk its white colour and also allows them to be separated from whey proteins at comparatively low centrifugal forces (~ 50 000 x g).

Table II-3. General properties of bovine casein micelles[1].

Property	*Unit*	*Value*
Compositional		
Water	% (w/w)	63
Protein	"	34.5
Minerals	"	2.5
Calcium	"	1.1
Magnesium	"	0.04
Phosphate	"	1.1
Citrate	"	0.14
Mean # casein monomers micelle^{-1}	-	~ 5000
# Micelles mL^{-1} milk	-	10^{14} - 10^{16}
Physico-chemical and hydrodynamic		
Mean molecular weight	Da	5.2 x 10^{8}
Diameter	nm	20 - 600
Mean free distance	nm	240
Isoelectric pH	-	4.6
ζ-potential	mV	- 8
Mean sedimentation coefficient	s x 10^{-13}	~ 500
Density	g mL^{-1}	1.063
Voluminosity	mL g^{-1}	4.4
Intrinsic viscosity	mL g^{-1}	10.11

[1]: Adapted from Schmidt *et al.* (1973), Dewan *et al.* (1974) and McMahon and Brown (1984)

Pertinent to any convincing model of casein micelle structure is that it must be able to account for the known physico-chemical properties of the casein proteins in milk. These include the colloidal stability of the caseins at the high calcium ion activity of milk, their high heat stability, and ability to be coagulated by acid, ethanol or chymosin. A successful model must also account for the ability of κ-casein to stabilize the other caseins against calcium-induced precipitation and take into consideration the fact that a large fraction of milk salt is associated with the caseins. The 'subunit' model for casein micelle structure, as proposed initially by Morr (1967), and refined by Slattery and Evard (1973), Slattery (1979), Schmidt (1980, 1982), Walstra and Jenness (1984), Whitney (1988) and Walstra (1990), agrees most closely with experimental observations, and shall be given the greatest emphasis here. For an historical overview of other models of the casein micelle, which are now considered ignominious, see Schmidt (1982) and Rollema (1992).

The fundamental assertion of all subunit models, which has been amply vindicated by experimental studies, is that the density of protein throughout the casein micelle varies and the micelle is composed of distinctive subunits or 'submicelles'. The high voluminosity and hydration of the casein micelle (McMahon and Brown, 1984) indicate a high porosity and hence unhomogenous structure. The use of small-angle neutron scattering (Stohart, 1989) or small-angle X-ray scattering (Kumosinski *et al.*, 1988) to probe the internal structure of the casein micelle has also revealed the presence of subunits. Electron microscopy (Buchheim and Welsch, 1973; Knoop *et al.*, 1973; Kalab *et al.*, 1982) has shown that a casein micelle consists of compact, spherical protein domains, which are joined together to form a porous network (Figure II-2).

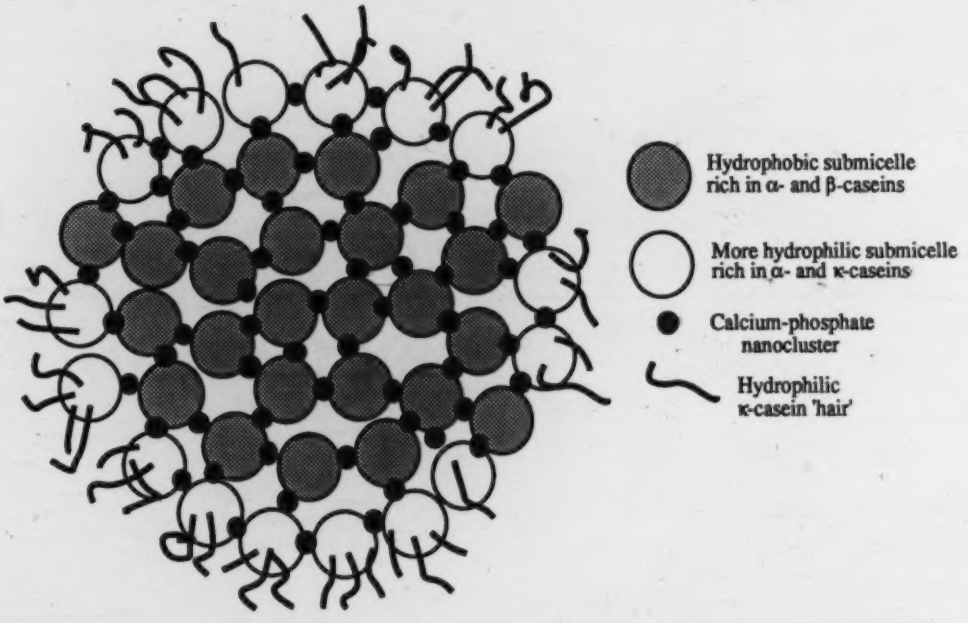
Hydrophobic submicelle rich in α- and β-caseins
More hydrophilic submicelle rich in α- and κ-caseins
Calcium-phosphate nanocluster
Hydrophilic κ-casein 'hair'

Figure II-2. Highly schematic illustration of the subunit model for the structure of a casein micelle.

Electron microscopy has revealed that the subunits are spherical and have diameters in the range 10 - 20 nm, though the reliability of electron microscopy for the estimation of size is open to question (Buchheim and Welsch, 1973). A typical submicelle would probably contain 15 - 25 casein monomers (Walstra, 1990) and a typical casein micelle would be composed of ~ 250 subunits. The colloidal minerals of the casein micelle

probably occur in the form of spherical granules or calcium phosphate 'nanoclusters' of ~ 2.5 nm diameter (Holt and Hukins, 1991). These nanoclusters are probably attached to the phosphoseryl residues of the caseins, located on the surface of the submicelles, allowing the submicelles to be crosslinked into a network. Submicelles have been shown also to exist independently in milk, where they account for ~ 80% of the total number of particles containing casein, but this is equivalent to only about 3% by volume of the casein fraction (Schmidt *et al.*, 1973).

Two types of subunits are postulated to occur in the micelle - those with or without κ-casein (Walstra, 1990). Subunits located at the surface of the micelle contain almost equimolar amounts of κ-casein and the α-caseins, while those located in the interior of the micelle have equimolar amounts of the α-caseins and β-casein, with possibly a small amount of κ-casein (Dalgleish *et al.*, 1989). The distribution of the caseins within the subunits is uneven. It is thought that all of the subunits have a hydrophobic core surrounded by the relatively hydrophilic sections of the caseins. In the case of interior subunits, the hydrophilic sections of the α- and β-caseins (more specifically, those regions rich in phosphoseryl residues) are complexed to the calcium phosphate nanoclusters. Submicelles located at the surface of the casein micelle contain a hydrophobic core consisting mostly of the α-caseins. The surface part of the subunit exposed to the solvent is coated with κ-casein, while the surface part orientated towards the interior of the micelle probably contains hydrophilic sections of the α-caseins bound to the other subunits via calcium phosphate nanoclusters.

In milk, the calcium-sensitive caseins are rendered stable to calcium by κ-casein. Essentially, κ-casein shields the calcium-sensitive caseins from the aqueous environment and thus prevents their precipitation at the relatively high calcium ion activity. During the synthesis of casein micelles in the golgi apparatus of the mammary cells, the calcium-sensitive caseins undergo a limited degree of complexation; hydrophobic interactions lead to the formation of submicelles, which is followed by association of the submicelles into a network, modulated by calcium phosphate nanoclusters. The growth of the hydrophobic interior of the casein micelle is probably terminated when it becomes coated with κ-casein rich submicelles; this hypothesis is substantiated by the fact that there is a strong negative correlation between the κ-casein concentration in milk and average casein micelle size (McGann *et al.*, 1980; Donnelly *et al.*, 1984). In the absence of κ-casein, association of

calcium-sensitive caseins would be expected to proceed indefinitely, leading ultimately to their precipitation.

Coalescence between the casein micelles upon collision is prevented by the existence of κ-casein rich regions at the micelle surface; this occurs despite the close intermicellar proximity and hence high collision frequency. ^{1}H-nuclear magnetic resonance spectroscopy indicates that the C-terminal part of κ-casein, starting at amino acid residues 86 - 96, protrudes directly into the solvent (Griffin and Roberts, 1985; Walstra, 1990). The C-terminal end is flexible and has a hydrodynamic thickness of ~ 7 nm (Walstra, 1990) causing the surface of the casein micelles to display a 'hairy' conformation. At the normal (~ 6.7) pH of milk these 'hairs' are negatively charged, and are responsible for the micellar ζ-potential of - 8 mV, as measured by Schmidt and Poll (1986). Thus, electrostatic repulsion contributes to the colloidal stability of the micelles. However, calculations have show that these forces alone would be inadequate to prevent coalescence of the micelles (Walstra, 1990). Extra stability is provided by steric repulsion, a consequence of the protrusion of the C-termini of κ-caseins into the solvent. Steric repulsion occurs, in part, because if two 'hairs' come in close proximity their motility and hence entropy is reduced, which is a thermodynamically unfavourable situation (Walstra, 1990). Additional steric stability is provided by osmotic repulsion (Walstra, 1990).

The vital role of colloidal salts in maintaining micellar integrity has been well demonstrated; disruption of the structure of calcium phosphate nanoclusters by acidification (Dalgleish and Law, 1988, 1989; Law, 1996), dialysis (Holt *et al.*, 1986) or addition of the calcium-chelating agent EDTA (Griffin *et al.*, 1988), leads to disintegration of micelle structure. The precise structure of the calcium phosphate nanoclusters is not known with certainty, but the most recent evidence indicates that the structure is likely to resemble that of the dicalcium phosphate salt, $Ca_2HPO_4.H_2O$, also know as the mineral brushite (Holt and Hukins, 1991; Holt, 1995). It is thought that the dicalcium phosphate exists in the form of spherical nanoclusters of diameter ~ 2.5 nm, which are cross linked to the caseins via phosphoseryl groups. Further details on the structure of the colloidal milk salts will be discussed in section 2.1.

A model of the structure of the casein micelle proposed by Holt and Dalgleish (1986) was recently re-emphasized by Holt and Horne (1996) as a possible alternative to the subunit model. The model envisages the casein micelle as a tangled network of casein

monomers, cross-linked by calcium phosphate nanoclusters, in which the core and surface regions of the micelle have a similar distribution of casein types, and there is an absence of discrete subunits. The surface of the micelle contains protruding κ-casein 'hairs', which impart electrostatic and steric stability, but only about 10% of total κ-casein contributes to the formation of 'hairs'. A major difficulty with this model of casein micelle structure is that it contradicts electron microscopical data, which have shown the presence of a subunit structure.

The casein micelle is considered to have a number of biological functions (Holt and Hukins, 1991; Sawyer and Holt, 1993). The gel-forming capacity of casein micelles in the stomach of the calf, due to the action of chymosin, increases the residence time of casein in the digestive tract and thereby may regulate its hydrolysis into nutritionally-valuable free amino acids and peptides. These may well include various kinds of bioactive peptides, including β-caseinomorphins, which may be necessary to promote proper brain cell growth (Fox and Flynn, 1992) or stimulate the immune system (Migliore-Samour *et al.*, 1989). Another function of the casein micelle, as proposed by Sawyer and Holt (1993), could be to prevent ectopic calcification of the mammary gland. During milk biosynthesis, calcium, phosphorus and other minerals are actively transported from the blood into the mammary cells and if the intracellular concentration of these minerals is too high, they would precipitate leading to cell damage - otherwise referred to as ectopic calcification. The open conformations of the caseins and multiple phosphorylation cause them to bind to calcium and phosphorus and restrict the growth of calcium-phosphate nuclei. The high proline content of the caseins may also assist in the binding of calcium; Williamson (1994) pointed out that proline tends to restrict the conformational mobility of proteins, leading to a reduction in the unfavourable loss of entropy upon binding to various low MW species, especially if binding occurs in the vicinity of propyl residues. The high binding affinity of the caseins for calcium may not only prevent ectopic calcification, but also allows milk to contain a high concentration of calcium and phosphorus, required to meet the nutritional needs of the neonate.

1.2 The whey proteins

The whey or serum proteins comprise about 20% of the total protein in bovine milk. The most abundant whey proteins are β-lactoglobulin, α-lactalbumin, bovine serum albumin, the immunoglobulin fraction and proteose peptone 3; some properties of these proteins are shown in Table II-4.

Table II-4. Some physico-chemical characteristics of bovine whey proteins[1].

Protein	*Molecular weight (Da)*	*Mean hydrophobicity (kJ residue^{-1})*	*Isoionic pH*	*Sulphydryl groups*	*Genetic variants*
β-Lactoglobulin	18 362	5.03	5.26	5	A, B, C, H, W, D_a
α-Lactalbumin	14 174	4.68	4.74	8	A, B, C
Bovine serum albumin	66 267	4.30	5.13	35	One
Immunoglobulins					
SIgA	385 000 - 430 000	–	–	10	Numerous[3]
IgE	~ 150 000[2]	–	–	4	"
IgG_1	143 000 - 146 000	–	–	8	"
IgG_2	146 000 - 154 000	–	–	8	"
IgM	1030 000	–	–	22	"

[1]: Adapted from Eigel *et al.* (1984), Hambling *et al.* (1992) and Swaisgood (1992) where more detailed information is available.
[2]: Estimate based on the fact that IgE is a monomer and would be expected to have a similar molecular weight to IgG_1 or IgG_2.
[3]: Extreme genetic variability arises because of the numerous specificities needed for complexation with various species of antigens.

In contrast to the caseins, whey proteins exhibit globular conformations with well-defined tertiary structures, greater hydrophilicity, less amphipathicity, a more limited tendency for self-association, greater heat sensitivity, but less sensitivity to changes in ionic strength and pH. Most of the whey proteins contain thiol groups which can participate in sulphydryl-disulphide interchange reactions; this has a strong effect on the physico-chemical and functional properties of the whey proteins (Kinsella and Whitehead, 1989).

A number of studies have attempted to determine the secondary and tertiary conformations of the whey proteins and the results are much more consistent, as compared to reported structures for the caseins (Sawyer and Holt, 1993). Techniques such as Raman spectroscopy, optical rotary dispersion and circular dichroism have proved quite successful in determining the secondary structure of the whey proteins, as these methods are most

applicable to globular proteins. Additionally, albeit with some difficulty, it has been possible to prepare crystals of some of the whey proteins and hence determine their three-dimensional structures by X-ray crystallography.

1.2.1 β-Lactoglobulin

The most abundant protein in whey, β-lactoglobulin (β-lg), which comprises about 50% of the total serum protein in bovine milk, has been extremely well characterized; the structural and physico-chemical properties of this protein were reviewed by Hambling *et al.* (1992). At least six genetic variants of β-lg have been discovered in bovine milk (Hambling *et al.*, 1992; Ng-Kwai-Hang and Grosclaude, 1992); these are A, B, C, H, W and D_R and the primary structures of most of these variants are known. The A and B polymorphs are the most common in the milk of western breeds of cattle, occurring with almost equal frequency. Native β-lg contains two disulphide bonds (at cys_{66}-cys_{160} and cys_{106}-cys_{119} or cys_{106}-cys_{121}) and a free thiol group (at cys_{121} or cys_{119}). The free thiol is equally distributed at positions 119 and 121; a disulphide bridge joins residues 106 and 121, when the free thiol occurs at residue 119, and links residues 106 and 119 when the free thiol is at position 121. The other disulphide bridge always occurs between residues 66 and 160. The presence of disulphides and of a single sulphydryl group have an important bearing on the structure, reactivity and functionality of β-lg.

The secondary structures of β-lg have been calculated from circular dichroic and infrared spectroscopic data, which indicate that it contains ~ 10% α-helix, ~ 50% β-sheet and ~ 40% unordered structure (Timasheff *et al.*, 1966; Townend *et al.*, 1967; Rüegg *et al.*, 1975; Creamer *et al.*, 1983). Determination of the tertiary structure of β-lg has been complicated by the fact that at least 12 crystal forms of the protein have been isolated, depending on conditions of pH, ionic strength and the presence of ligands (Hambling *et al.*, 1992). Using low resolution (6 Å) X-ray crystallography, Green *et al.* (1979) revealed that a monomeric form of β-lg, isolated as four distinct crystal states, was roughly spherical with a diameter of 3 nm. Papiz *et al.* (1986), deduced from X-ray crystallographic data, at 2.8 Å resolution, a detailed three-dimensional structure for one of the crystalline states of β-lg. The structure contained 9 strands of anti-parallel β-sheet, 8 of which were involved in the formation of a barrel-like structure in the interior of the molecule; the barrel-like structure probably allows β-lg to bind hydrophobic molecules.

The A, B and C polymorphs of β-lg exhibit pH-dependent polymerization reactions (Creamer *et al.*, 1983; Pessen *et al.*, 1985; Casal *et al.*, 1988; Macleod *et al.*, 1995). At the normal pH (~ 6.7) of milk, β-lg exists as a dimer (MW 37 200 Da), whereas from pH 3.7 - 5.1 it forms octamers (MW 148 800 Da) or possibly higher order structures. At pH > 8.0 or pH< 2.0, β-lg dissociates into a monomer (MW 18 600 Da). The dimeric form of β-lg is stabilized by electrostatic interactions between asp_{130} and glu_{134} of one monomer with lys_{135}, lys_{138} and lys_{141} residues of another monomer (Creamer *et al.*, 1983). All of the pH-induced transitions of β-lg are freely reversible, except at pH > 8.0, where the monomeric form undergoes irreversible denaturation (Casal *et al.*, 1988).

Patocka and Jelen (1991a) reported that β-lg was capable of binding calcium to a limited extent, where protein bound calcium was determined by measuring the difference between the concentration of calcium in an ultrafiltration permeate and an ultrafiltration retentate. These workers found that, at an ionic strength of 0.08, calcium could only bind to β-lg at > pH 5, above which the protein had an increased affinity for calcium; a maximum binding capacity of 1.38 mol calcium mol^{-1} of protein occurred at pH 7, the highest pH used in this study. Since β-lg is not generally acknowledged to be a true metalloprotein, it is likely that calcium binding takes place at surface groups of the molecule and has a relatively limited impact on its tertiary structure.

Human plasma retinol-binding protein and bovine β-lg display high conformational homology, particularly with regard to their hydrophobic cores, which enable both proteins to bind to retinol. The conformational and chemical similarities between β-lg and retinol binding protein have prompted speculation that the biological function of β-lg is to protect retinol from oxidation and assist in its transport in into the blood of the neonate, via specific receptors in the intestine (Papiz *et al.*, 1986). Macleod *et al.* (1996) observed that hydrolysis of β-lg by trypsin was inhibited severely when β-lg was bound to retinoic acid; thus, it can be speculated that limited hydrolysis of β-lg in the intestine occurs to generate biologically active peptides.

1.2.2 α-Lactalbumin

The second most abundant serum protein is α-lactalbumin (α-la), constituting ~ 20% of the total whey protein fraction. Like β-lg, the structural and physico-chemical properties of α-la have been characterized in great detail and comprehensive reviews are available

(Kronman, 1989; Brew and Grobler, 1992). Three genetic variants of α-la, A, B and C, are known to occur in bovine milk (Ng-Kwai-Hang and Grosclaude, 1992) with the B variant being the most common. The primary structures of the A and B variants have been determined (Swaisgood, 1982; Eigel *et al.*, 1984). High levels of tryptophanyl and aspartic acid residues, the presence of single arginine and methionine residues, four intra-chain disulphide bonds and the absence of phosphoryl and free sulphydryl groups are distinguishing features of this molecule. The protein has a limited tendency for self-association, even when subjected to prolonged heating (Chaplin and Lyster, 1986).

Circular dichroic spectra show that at the normal pH (~ 6.7) of bovine milk, the secondary structures of α-la consist of ~ 26% α-helix, ~ 14% β-sheet and ~ 60% unordered structure (Creamer *et al.*, 1983). The monomeric form of α-la is a compact, globular, roughly spherical protein. Sequence homology between α-la and egg white lysozyme, especially with respect to the location of thiol groups, suggests that the two proteins have similar three-dimensional structures. Therefore, attempts to evaluate the tertiary structure of bovine α-la have been based on the known structure of lysozyme, as determined by X-ray crystallography (Browne *et al.*, 1969; Warme *et al.*, 1974) and are in good agreement with the structure of baboon α-la, as determined by high-resolution (1.7 Å) X-ray crystallography (Acharya *et al.*, 1989).

α-La is a calcium-binding protein, which is also capable of binding manganese, potassium, sodium, terbium, zinc and possibly other metals; the metal ion binding properties of α-la were surveyed by Kronman (1989) and Brew and Grobler (1992). The single calcium ion is bound in a pocket surrounded by seven ligands between residues 79 and 88 (Acharya *et al.*, 1989). The presence of calcium is essential to the maintenance of the native structure of α-la, indicating that α-la is a true metalloprotein. Rao and Brew (1989) showed that α-la in the reduced (where intramolecular thiol bonds are broken), denatured state, exhibited 90 - 100% renaturation in the presence of calcium, when the free thiol groups were oxidized; however, in the presence of EGTA, a calcium chelator, only about 2% of α-la renatured upon oxidation. At pH 4.0, protonation of α-la causes the release of the calcium ion, which causes the molecule to assume the molten globule state (a loosening of conformation, but without loss of secondary structure, see section 3.1), also known as the 'A' state (Desmet *et al.*, 1987; Alexandrescu *et al.*, 1993; Chyan *et al.*, 1993); this causes a sharp decline in heat stability (Bernal and Jelen, 1984).

It has been shown that in model solutions α-la exhibits self-association reactions. At pH < 4.0, the sedimentation constant of α-la was in the range 10 - 14 S, compared to a value of 1.93 S for the monomer, and the extent of association at pH < 4.0 was enhanced when pH, temperature, ionic strength, protein concentration and time were increased (Kronman and Andreotti, 1964; Kronman *et al.*, 1964). On the alkaline side of the isoelectric point, α-la was also shown to undergo self-association, but to a much lesser extent. The tendency of α-la to self-associate at < pH 4.0 indicates a greater susceptibility to intermolecular interaction when it is converted to the molten globule state. This could also explain the increased viscosities of solutions of whey protein concentrate at acidic pH, observed by Rattray and Jelen (1995).

The biological function of α-la is to act as a co-enzyme for galactosyl transferase (GT). This enzyme is found in the golgi bodies of a range of secretory cells, including the mammary cells, and is involved in the synthesis of glycoproteins and various di- and oligosaccharides, which contain galactose. In the absence of α-la, GT has a poor binding affinity for glucose, but by complexing with α-la its affinity for glucose is increased ~ 1000 fold (Bell *et al.*, 1976), enabling synthesis of lactose in the golgi bodies of mammary cells. The synthesized lactose accumulates in the lumen of the golgi body and generates an osmotic potential causing a flow of cellular water into the lumen; therefore, α-la indirectly controls the water content of milk. The molecular details of binding between α-la and GT have been described by Kronman (1989) and Brew and Grobler (1992); of note is the fact that calcium is essential for maintenance of the native structure of α-la and hence complexation between the α-la and GT.

1.2.3 Bovine serum albumin

Bovine serum albumin (BSA) represents ~ 10% of the serum protein in bovine milk and is identical to blood serum albumin. Only one genotype of BSA has been identified in bovine milk. Its primary structure was determined by Reed *et al.* (1980); it contains 582 amino acid residues, giving a calculated MW of 66 267 Da, 17 intramolecular disulphide bonds and one free sulphydryl group at residue 34. Using circular dichroism Reed *et al.* (1975) estimated that ~ 55, 16 or 29% of the amino acid residues of bovine BSA occur as α-helical, β-sheet or unordered structures, respectively; the integrity of these structures was maintained upon hydrolysis of the protein into 12 fragments by pepsin or trypsin. The

numerous disulphide bonds would be expected to impose conformational restrictions on the molecule, contributing to a compact shape. Isolation of crystals of BSA has proved difficult and therefore the tertiary structure has not been determined by X-ray crystallography. However, Carter *et al.* (1989) produced an electron density map for human serum albumin, which would likely exhibit a structure similar to BSA. This structure showed the presence of large (~ 9 nm x 9 nm) solvent channels within the molecule, which concurred with the high hydration value (30 g H_20 100 g^{-1} protein) for BSA. Hydrodynamic data indicate that BSA is a prolate ellipsoid with dimensions of ~ 4 x 14 nm (Wright and Thompson, 1975).

The protein has been shown to bind calcium and other types of metal ions (Kronman, 1989). Five to twelve binding sites for calcium have been identified at neutral pH, but it is not well known how important calcium is in the maintenance of native structure. The hydrophobic nature of the interior of BSA allows it to strongly bind free fatty acids; a biological function of blood serum albumin is in the transport of free fatty acids through the blood circulatory system. No specific biological function has been identified for BSA in milk and it could merely be a 'leakage' protein transferred incidentally from the blood.

1.2.4 Immunoglobulins

The immunoglobulin (Ig) fraction of bovine milk is a complex mixture of large glycoproteins which exhibit antibody activity. Their structures, mode of synthesis and transport and role in immunology have been reviewed by Nezlin (1977) and Larson (1992). The Ig's are synthesized by plasma cells, which are located throughout the body including the blood and the mammary gland. The plasma cells evolve from antigen-stimulated B-lymphocytes; in turn, the B-lymphocytes are synthesized in the bone marrow (Larson, 1992). The Ig's occur in bovine milk at low concentrations (0.06 - 0.10%, w/v) but are found in colostrum at much higher levels where they may constitute 80% of the whey protein fraction (Smith, 1946). Antibodies exhibit the most extreme variability of any protein class; about a million types of antibodies probably exist (Nezlin, 1977). Nevertheless, antibodies can be separated into fundamental classes; in bovine milk at least five classes of Ig have been recognized, namely, IgA, IgE, IgG_1, IgG_2 and IgM. In bovine

colostrum or milk, IgG_1 is by far the most abundant Ig, comprising about 85% or 66% of the total Ig fraction, respectively (Larson, 1992).

All classes of Ig exist as either monomers or polymers of a basic subunit, which is composed of four polypeptide chains linked covalently by disulphide bridges (Figure II-3).

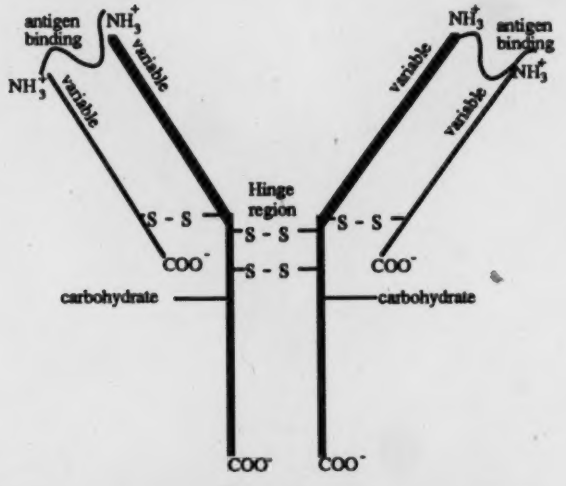

Figure II-3. Diagrammatic representation of the building block of an immunoglobulin. The heavy or light chains are indicated by thick or thin lines, respectively.

Two of the polypeptide chains are heavy (MW ~ 50 000 - 70 000 Da) while the other two are light (MW ~ 20 000 Da). Two types of light-chain, κ and λ, occur in all Ig classes, but in different ratios for each class, and any individual Ig molecule contains only κ or λ. Each class of Ig has a distinctive heavy chain designated γ (γ_1, γ_2, γ_3, γ_4) in IgG, α (α_1, α_2) in IgA, μ in IgM, δ in IgD and ε in IgE. The carbohydrate moiety is attached to each of the two heavy chains of the monomer, approximately a third of the way up from the carboxyl end of the chains. The C-terminal half of both the heavy and light chains, for all classes of Ig, is highly conserved, while the N-terminal half displays considerable variability and hence accounts for Ig specificity against antigens. The monomeric unit of each Ig can bind to two antigens, as indicated in Figure II-3, and ~ 110 - 120 amino acid residues are involved in the binding of an antigen. The 'hinge' region of the monomer is rich in propyl residues, which contribute to Ig flexibility, the purpose of which is probably to facilitate the binding of large antigens.

The basic properties of the Ig's were summarized by Larson (1992). IgE, IgG_1 and IgG_2 are monomers, consisting of a single Ig unit, the latter two containing ~ 2 - 4% carbohydrate. IgA is a dimer, with ~ 8 - 9% carbohydrate; two IgA monomers bind to a protein called J-protein (MW ~ 16 000) and a secretory protein (MW ~ 75 000), to form a complex called SIgA. The secretory protein, initially located on the epithelium of the mammary cell, is involved in the transport of IgA into milk. The secretory protein also occurs in milk in the free state at an appreciable concentration. IgM contains ~ 12% carbohydrate; it consists of a pentamer of five disulphide-linked monomeric Ig units, all of which are also joined to J-protein. A monomeric Ig, called IgD, has been detected in human milk but it is not known if it occurs in bovine milk.

The biological function of the bovine Ig fraction is to confer passive immunity *via colostrum* to the neonate shortly after birth. The absorption of Ig in the intestine of the neonate occurs for only about a day, after which the intestinal cells undergo closure. Ruminants (cow, goat, sheep) must obtain their Ig *via colostrum*, whereas other species of mammal (guinea pig, human, kangaroo, rabbit) obtain Ig *in utero* or by a combination of the two mechanisms (cat, dog). Although Ig's make up ~ 10% of total whey protein in milk, their contribution to the functional properties of milk and dairy products has been largely ignored.

1.2.5 Lactoferrin

Lactoferrin, also known as lactotransferrin, occurs in bovine colostrum at a concentration of ~ 1 g L^{-1} or 0.02 - 0.35 g L^{-1} in bovine milk (Reiter, 1985); the level in human milk is much higher (> 2 g L^{-1}). The protein has a MW of 76 500 Da and contains two carbohydrate groups and 17 disulphide bridges (Castellino *et al.*, 1970). Two ferric (Fe^{3+}) ion-binding sites, which also bind to bicarbonate ion (HCO_3^-), have been identified on the molecule (Metz-Boutique *et al.*, 1984), corresponding to domains with similar conformations. The iron-binding ability of lactoferrin has been shown *in vitro* to confer bacteriostatic properties to the molecule (Reiter, 1985), leading to speculation that the function of this protein is to help protect against the growth of undesirable bacteria in the intestine of the neonate. In the native state, lactoferrin is only partly (8 - 30%) saturated with iron, indicating a capacity to chelate iron and render it unavailable to micro-organisms. A lactoferrin receptor has been located in the brush border region of the small intestine of

the rabbit, which suggests that lactoferrin may also regulate the transport of iron into the bloodstream, though it is not certain if this involves promotion or inhibition of iron transport (Mazurier *et al.*, 1985).

1.2.6 Folate -binding proteins

A number of different types of folate-binding protein (FBP) occur in a variety of tissues both extra- and intracellularly (Cremin and Power, 1985; Fox and Flynn, 1992). The FBP of bovine milk occurs at a trace concentration, consistent with the low concentration of folic acid, the latter determined as ~ 50 - 60 $\mu g\ L^{-1}$ milk (Cremin and Power, 1985). The FBP contains 222 amino acid residues, has a MW of ~ 30 000 Da and contains ~ 10% carbohydrate (Fox and Flynn, 1992). The concentration of FBP in milk exceeds that of folate (Cremin and Power, 1985), indicating that all of this vitamin is bound to FBP. The physiological function of FBP in milk may be to regulate the transport of folate to the neonate; Colman and Herbert (1980) showed that the small intestine contains receptor sites for FBP. However, it is not clear whether the purpose of FBP is to actively promote the transport of folate across the small intestine or to regulate and possibly even slow down its transport (Fox and Flynn, 1992). In human milk, Ford *et al.* (1977) speculated that the binding of folate to FBP reduces the availability of folate to undesirable intestinal bacteria.

1.2.7 Vitamin B_{12}-binding protein

It has been shown that in bovine milk, almost all of the vitamin B_{12} is bound to vitamin B_{12}-binding protein (B_{12}BP) (Gregory, 1954). Milk contains ~ 4 $\mu g\ L^{-1}$ of vitamin B_{12} (Cremin and Power, 1985), indicating that B_{12}BP probably also occurs at a trace concentration in milk. Burger and Allen (1974) showed that B_{12}BP in human milk had a MW of 61 000 - 63 000 Da and contained ~ 35% carbohydrate. The B_{12}BP may be involved in sequestering vitamin B_{12} from the blood into the mammary cells and hence into milk. It may also inhibit the growth of undesirable bacteria in the intestine of the neonate by reducing the bioavailability of vitamin B_{12} (Cremin and Power, 1985).

1.2.8 β_2-Microglobulin

The protein β_2-microglobulin occurs in a variety of tissues, where it forms part of a cell glycoprotein complex, referred to as histocompatability antigens, which are integrated into

the cell membrane (Groves and Greenberg, 1982). Histocompatability antigens are involved in cell recognition and probably play a role in the regulation of many diseases. Apparently, the free β_2-microglobulin that occurs in bovine milk represents a 'leakage' protein, having no known biological function in milk. The structure of β_2-microglobulin is similar to the conserved portions of the heavy or light chains of IgG. The protein contains 98 amino acid residues, giving a MW of 11 630 Da (Groves and Greenberg, 1982). β_2-Microglobulin displays self-association reactions, the extent of which increases with increasing protein concentration (Groves and Greenberg, 1982).

1.2.9 Proteose peptones

The proteose peptone (PP) fraction of bovine milk is a heterogeneous mixture of polypeptides, of which many are phosphorylated and are the result of proteolysis of the caseins by plasmin, an indigenous milk enzyme. Knowledge on the PP fraction was synopsized by Grufferty and Fox (1988) and Pâquet (1988). At least 38 PP components have been identified in bovine milk, of which 25 are due to hydrolysis of the caseins by plasmin (Andrews and Alichandis, 1983); of these 25 PP's, ~ 52% were derived from β-casein, ~ 29% from α-$_{s1}$-casein, ~ 9% from α-$_{s2}$-casein and ~ 4% from κ-casein. Schaar (1985) estimated that ~ 23% of the PP's were the consequence of post-secretory plasmin activity, whereas the remainder were generated within the milk secretory cell. The PP fraction cannot be strictly deemed as belonging to the serum or casein fraction of milk; on the one hand many or possibly all of them are soluble at pH 4.6, but on the other hand, most are derivatives of the caseins.

Hydrolysis of β-casein by plasmin generates at least 6 different kinds of polypeptide (Pâquet, 1988), all of which have been isolated from bovine milk: γ_1-casein (β-casein fragment 29-209), γ_2-casein (β-f 106-209), γ_3-casein (β-f 108-209), PP8 fast (β-f 1-28), PP8 slow (β-f 29-105/107) PP5 (β-f-1-105/107). The γ-caseins are associated with the casein micelle, while the PP's are in equilibrium between the serum and micellar phases. In model systems, plasmin is able to cleave α-$_{s1}$-casein at 7 sites (McSweeney *et al.*, 1993), but in milk the degree of hydrolysis is probably much less, considering that much of the α-$_{s1}$-casein is 'buried' in the interior of the casein micelles and therefore would be inaccessible to plasmin. At least 8 sites of α-$_{s2}$-casein can be hydrolyzed *in vitro* by plasmin to generate 10 species of polypeptide (Le Bars and Gripon, 1989), but the extent

of hydrolysis and the resultant polypeptide fractions which may occur in bovine milk are not known. Plasmin is inactive against κ-casein, α-la and β-lg. Moreover, the thermal aggregation of β-lg onto casein micelles inhibits the hydrolysis of the β- and α-caseins by plasmin (Grufferty and Fox, 1986).

The most abundant type of PP in milk is PP3, which represents ~ 25% of total PP, equivalent to a concentration of ~ 300 mg L^{-1}. However, this PP does not originate from hydrolysis of any casein type and can be considered as a genuine whey protein. The PP3 component contains 135 amino acid residues and has a MW of ~ 28 000 Da; the high MW has been attributed to 3 glycosylation sites on the molecule at thr_{16}, thr_{86} and asn_{77}, which contain glucosamine and/or galactosamine (Sørensen and Petersen, 1993). The protein shows variable phosphorylation, with up to 5 phosphoseryl residues per molecule. A fragment (f54 - 135) of PP3 has been isolated from bovine milk, the existence of which was attributed to the action of plasmin (Sørensen and Petersen, 1993). Antibodies against milk-fat-globule membrane proteins are known to bind to PP3, prompting speculation that PP3 is also found in the fat globule membrane; however, on the basis of its relatively low hydrophobicity, it is difficult to envisage how PP3 could integrate into the fat-globule membrane, which led Sørensen and Petersen (1993) to suggest that PP3 and fat-globule membrane proteins possess similar epitopes for the binding of antibodies. No biological function has been identified for PP3, though it has been suggested that its purpose could be to help prevent the precipitation of calcium in milk and in the mammary cells, its multiple phosphorylation enabling it to bind strongly with calcium (Sørensen and Petersen, 1993).

2 The milk salt system

The protein and fat components of bovine milk have been the subject of numerous investigations, which may have been stimulated by the fact that their manipulation is the basis of the manufacture of most dairy products. The importance of the milk salt system should not be underestimated either, especially in view of the fact that much of the physico-chemical behaviour of milk proteins is influenced by ionic conditions; however, comparatively little research has been carried out on the milk salt system. An improved understanding of the milk salt system is desirable because of its relevance to the heat stability of milk (McCrae and Muir, 1995) or concentrated milk (Singh *et al.*, 1995), its role in the coagulation of enzymatically-treated casein micelles (Lucey and Fox, 1993) and

its contribution, especially that of calcium and phosphorus, to the nutritional quality of milk (Flynn and Power, 1985). Reviews on the milk salts have been written by Pyne (1962), Walstra and Jenness (1984) and Holt (1985, 1995), from these it is evident that the topic is rather complex and incompletely understood.

Normal bovine milk contains ~ 160 mmol L^{-1} of total salts, of which ~ 35 mmol L^{-1} are colloidal, being associated with casein and to a much lesser extent with whey proteins, and ~ 125 mmol L^{-1} are dissolved in the aqueous phase of milk (Holt, 1985); on a mass basis, this corresponds to about one quarter colloidal and three quarters soluble salts. The distribution of the principal milk salts between the soluble and colloidal states is shown in Table II-5. It should be noted that the term 'inorganic phosphate' refers to phosphate that is not directly bound to the casein proteins; that is, it excludes the phosphate of the phosphoseryl groups of the caseins, which is referred to as organic phosphate, and can be considered as part of the casein protein.

Table II-5. The distribution of the principal minerals of milk[1] between the colloidal and soluble phases, shown on a molar or a mass basis.

Constituent	*Soluble*		*Colloidal*	
	mMolar	*mg 100 g^{-1} milk*	*mMolar*	*mg 100 g^{-1} milk*
Calcium	10	40	20.3	81
Magnesium	3.3	8	1.9	5
Inorganic phosphate	11.9	113	9.5	90
Citrate	9.1	175	1.0	19
Sodium	21.8	50	1.0	2
Potassium	37.5	146	2.2	9
Chloride	31.5	110	0	0
Total	125.1	643	35.9	206

[1]: Adapted from Holt (1985)

Milk is isoosmotic with the blood and its molality is primarily influenced by the concentration of lactose and soluble salts. A consequence of the isomolality of milk and blood is that the concentration of lactose and soluble salts are inversely proportional. The mode of secretion of the milk salts in the mammary gland is not completely understood, but

it is generally accepted that the milk salts are transferred from the blood into the cytosol and from there into the intravesicular fluid of golgi apparatus, where some salts remain soluble while others bind to the caseins causing them to associate into micelles (Holt, 1985). Sodium, chloride and potassium probably undergo passive transport from the blood, whereas the high concentrations of calcium, inorganic phosphate and citrate in the golgi apparatus implies that an active transport system exists. Some inorganic phosphate is also generated during the synthesis of lactose in the golgi body (Holt, 1985).

It is convenient to describe the states of the colloidal and soluble milk salts separately, though it must be emphasized that the two phases are strongly interrelated and changes in the composition of one phase, for example induced by changes in pH or temperature, usually lead to changes in the composition of the other (see section 2.3).

2.1 Colloidal milk salts

In discussions on the colloidal salt component of casein micelles, the terms 'colloidal calcium phosphate' (CCP) and 'micellar calcium phosphate' (MCP) are frequently encountered, which have been given various definitions in the literature. The term CCP frequently refers to all of the salts associated with the casein micelle, including organic and inorganic constituents, and in addition to calcium and phosphorus, also citrate, magnesium and other minerals. A number of definitions exist for the term MCP, some of them confusing and rather superfluous, and contributing little to an improved understanding of the nature of the colloidal minerals. In some articles, it appears that MCP refers to the entire mineral entity associated with casein micelles and is therefore equivalent to CCP. On the other hand, van Dijk (1990) defined MCP as "the inorganic colloidal phosphate and that part of the divalent colloidal cations [Ca^{2+} and Mg^{2+}] that is assumed to be associated with inorganic colloidal phosphate". This author implied that the term MCP was useful because when all of the organic phosphate of casein was removed, most of the minerals dissociated and were termed 'MCP'; however, some citrate, calcium and magnesium were still bound to the micelle and as such could be differentiated from the 'MCP'. Nevertheless, it is apparent that the phraseologies used by van Dijk (1990) and other authors to define MCP are somewhat unwieldy and tend to impede the visualization of structures for the colloidal milk salt component. In the current treatise, the term CCP shall only be used and will indicate the entire mineral component of the casein micelle.

The structure of the CCP and its mode of attachment to the caseins has received considerable attention but the issue has not been satisfactorily resolved. A basic difficulty is the fact that calcium and phosphate can bind together to form a variety of salt species, all of which could conceivably exist in the CCP of milk (Holt, 1995). The various models proposed for CCP were summarized by van Dijk (1990), but most of these models are rather specious. The view that is now evolving is that CCP occurs in the form of discrete, spherical 'nanoclusters', which are attached to the phosphoseryl residues of the caseins.

The stochiometry of the CCP granules is not known with certainty, but small angle X-ray scattering (Holt and Hukins, 1991), has indicated that the structure of nanoclusters could be similar to that of brushite, a dicalcium phosphate salt ($CaHPO_4.2H_2O$). Further evidence for the existence of a dicalcium phosphate salt was provided by Chaplin (1984), who noted that if an equilibrium existed in milk between a solid and a soluble phase of calcium phosphate, then such an equilibrium could be characterized by a solubility product (k_{sp}), the value of which should be independent of pH. By measuring the concentration of ultrafilterable calcium and inorganic phosphate, it was possible to calculate k_{sp} for various forms of calcium phosphate. It was shown that the k_{sp} value for dicalcium phosphate in milk, did not vary over pH 4 - 8, whereas k_{sp} values for tricalcium phosphate or hydroxyapatite exhibited substantial variations. Similar results were obtained by Holt (1982, 1994) who showed that the CCP of bovine, caprine and human milk was likely to resemble dicalcium phosphate.

Electron microscopy has shown a diameter of ~2.5 nm for each CCP nanocluster (McGann *et al.*, 1983), indicating that it would contain about 66 calcium ions, 66 phosphate moieties and 132 water molecules, assuming that a brushite-like structure exists and that inclusion of the small amounts of colloidal citrate and magnesium would not alter the calculation appreciably (Holt and Hukins, 1991). The phosphate moieties would include about 22 phosphorylated amino acid residues of the caseins and since caseins on average contain 4 - 5 phosphate groups, ~ 5 casein molecules would be attached to each nanocluster (Holt and Hukins 1991). The intergranule distance was calculated to be 10.3 nm (Holt and Hukins, 1991), which is of a similar size to the diameter of a submicelle, sustaining the idea that a network of subunits is held together by calcium-phosphate nanoclusters, to make up the casein micelle.

The term colloidal minerals usually implies minerals associated with casein, but it should be pointed out that some of the whey proteins can also bind to minerals. These include the binding of calcium to α-la or β-lg or iron to lactoferrin, which was described in sections 1.2.1, 1.2.2 and 1.2.5 of this chapter. Compared to the caseins, the ability of the whey proteins to bind to metal ions is extremely limited and probably only one type of mineral at a time is involved in binding. Thus, in this sense the structure of the colloidal minerals of the whey proteins is relatively simple. However, the binding of metal ions has a profound effect on the tertiary structure of whey proteins, especially that of α-la and lactoferrin and of course the tertiary structure of a protein is a complicated attribute.

2.2 Soluble milk salts

The overall composition of the soluble milk salt system is known, as shown above in Table II-5, but it must be pointed out that individual ion species may exist freely or be combined with each other to generate a large variety of other ion species and it is not possible to measure directly the concentration of most of the soluble ion types which could occur in milk. Nevertheless, algorithms have been developed which attempt to predict the exact composition of the soluble milk salts; such procedures consider the principle of electroneutrality, the experimentally determined activities of a limited number of ions, such as Ca^{2+}, K^+, Na^+ or Cl^-, and the equilibrium constants between various soluble species. The results of one such attempt by Holt (1985) are shown in Table II-6.

Table II-6. Concentrations of soluble salt species in bovine milk.

Anion	*Free anion*	*Complexed to cation:*			
		Ca^{2+}	Mg^{2+}	Na^+	K^+
	Concentration (mM)				
$HCit^{2-}$	0.04	0.01	~0	~0	~0
Cit^{3-}	0.26	6.96	2.02	0.03	0.04
$H_2PO_4^-$	7.50	0.07	0.04	0.1	0.18
HPO_4^{2-}	2.65	0.59	0.34	0.39	0.52
PO_4^{3-}	~0	0.01	~0	~0	~0
Cl^-	30.90	0.26	0.07	0.39	0.68
Free cation		2.0	0.81	20.9	36.3

From this salt distribution, it can be seen that most of the Na^+, K^+ and Cl^- are found in the free state, but H^+, Ca^{2+}, Mg^{2+}, PO_4^{3-} and Cit^{2+} are mostly bound to each other to form various ionic species. Only about 20 - 30% of soluble Ca^{2+} or Mg^{2+} occur as free ions, as they are mostly bound to Cit^{3-}. Almost all of the citrate exists in bound form, as $CaCit^-$ or $MgCit^-$ and virtually all of the inorganic phosphate is bound to H^+ ions to form HPO_4^{2-} or $H_2PO_4^-$ some of which in turn binds to Ca^{2+} resulting in the formation of various soluble calcium phosphate species.

At natural pH, milk is highly supersaturated with monocalcium phosphate, marginally supersaturated with dicalcium phosphate and saturated or undersaturated with tricalcium phosphate (Holt, 1985). Therefore, it might be expected that the soluble milk salts would have a natural tendency to precipitate. This is indeed the case for milk ultrafiltrate which upon standing at room temperature develops a bluish tinge due to the growth of calcium phosphate (Holt, 1995). In milk itself, no such spontaneous precipitation occurs because an equilibrium exists between the calcium and phosphate ions in solution and the CCP. Therefore, the presence of casein in milk has a direct impact on the stability and structure of the soluble milk salt component.

2.3 Factors affecting the partitioning of milk salts

The distribution of milk salts between the colloidal and soluble states is affected by a number of factors, many of which are relevant to the processing of milk. These include changes in pH or temperature, concentration of milk by evaporation or ultrafiltration (UF), and the presence of various additives. In general, alterations in the partitioning of milk salts by these factors are due to altered solubilities of the CCP or the various soluble salt species. When the milk salt system is changed, structural changes in the casein micelles will result, due to the role of CCP in maintaining micelle structure.

2.3.1 pH

The addition of increasing amounts of acid to milk leads to a progressive dissolution of CCP and a concurrent increase in the concentration ([ ]) of soluble salts, especially the $[Ca^{2+}]$ and $[PO_4^{3-}]$ (Law, 1996). Very little information exists on the mechanism of dissolution of CCP, but this is presumably related to increased protonation of the caseins, perhaps especially at the phosphoseryl residues. It is also notable that at < pH 5, milk

becomes undersaturated with respect to mono-, di- and tricalcium phosphate, which probably provides a driving force for further dissolution of CCP. The inorganic phosphate and calcium released by dissolution of CCP probably do not exist entirely as free ions, but react with other ionic species to distort the composition of the soluble salt phase. In particular, released PO_4^{3-} ions combine with H^+ ions to form HPO_4^{2-} or $H_2PO_4^-$, which accounts for the high buffering ability of milk or more specifically the CCP of casein micelles. These species in turn can combine with Ca^{2+} to generate calcium phosphate salts, leading to a reduction in the activity of calcium ions (aCa^{2+}) (Holt, 1985).

The dissociation of CCP during the acidification of milk is probably irreversible. Lucey *et al.* (1993a, b, 1996) observed that, during the acidification of milk, maximum buffering capacity occurred at ~ pH 5.1, but upon neutralization of the acidified milk, maximum buffering capacity was shifted to ~ pH 6.3; this change indicated that when CCP was solubilized by acidification, it did not reassociate with the casein after subsequent neutralization. These authors speculated that at pH 5.1, the buffering agent was PO_4^{2-}, and at this pH its concentration was maximal, corresponding to complete solvation of CCP; when the acidified milk was neutralized, dissolved CCP did not reassociate with the casein micelles, with the result that at pH 6.3 $Ca_3(PO_4)_2$ formed with liberation of H^+ ions, which were responsible for the buffering action against the added OH^- ions.

Although acidification alters the amount of CCP in milk, it is possible that the structure of the residual CCP is not altered. Chaplin (1984) ultrafiltered milk, from pH 4 - 8, and reported that the ratio of calcium to inorganic phosphate in the ultrafiltrate was constant (~ 1.6), which may indicate that the dicalcium phosphate structure of the remaining indigenous CCP was preserved.

The acid dissolution of CCP causes dissociation of the caseins from the micelle. Roefs *et al.* (1985) found that, at low temperature (5 - 8°C), the concentration of non-sedimentable β- and κ-casein, and to a lesser extent the α-caseins, increased upon acidification to pH 5.4, where ~ 60% of total casein was solubilized, and casein micelle voluminosity was maximal. Similar results were reported by Dalgleish and Law (1988), who noted also that dissociation of the caseins was likely to proceed on a monomeric basis, rather than in the form of submicelles, because the ratio of the dissociated casein types did not correspond to the natural ratio in casein submicelles. Reducing the pH below 5.4 probably causes the dissociated caseins to aggregate and become co-sedimentable with the

micelles. At pH 4.6 and 20°C, isoelectric precipitation of the caseins occurs which may involve the formation of discrete particles, consisting of the caseins which had originally dissociated from the micelle (Mulvihill and Grufferty, 1995).

Very little is known about how alkalization affects the partitioning of the milk salt system. Holt (1985) pointed out that if the pH of milk was increased, then one would expect a shift from $H_2PO_4^-$ towards HPO_4^{2-} and hence towards PO_4^{3-}, which in turn might increase the tendency of Ca^{2+} to precipitate as $CaHPO_4$ or $Ca_3(PO_4)_2$. Lucey *et al.* (1993a) observed little change in the buffering index of milk when the pH was increased from 6.7 to 8.0, indicating minimal changes in milk salt partitioning.

2.3.2 Temperature

Several studies indicate that increasing the temperature of milk causes a shift from soluble to colloidal salt, which seems to involve predominantly the conversion of soluble monocalcium phosphate to insoluble di- and tricalcium phosphates. Pouliot *et al.* (1989a) reported that the concentration of calcium and inorganic phosphate in milk UF permeate exhibited a linear decrease when milk was ultrafiltered at increasing temperatures, from 4 - 90°C, indicating that the effect of heating was to reduce the proportion of milk salt in the soluble state. This was confirmed by Pouliot *et al.* (1989b) who showed also that cooling of milk, which had been heated, caused an almost complete restoration of the original salt equilibrium. Similarly, Geerts *et al.* (1983) found that when milk was heated at 115°C for ~ 1 -11 min, the aCa^{2+} decreased, but upon cooling there was a logarithmic increase in aCa^{2+}. Further evidence for the reversibility of heat-induced changes in salt equilibria was provided by Law (1996), who observed that the release of calcium and inorganic phosphate from casein micelles upon acidification was not altered after heating milk at 85°C for 10 min, followed by storage for 22 h at 4, 20 or 30°C. Likewise, Singh *et al.* (1996) reported that when milk was heated at 70 - 90°C for 5 min, the acid solubilization of CCP was not affected, as measured by an increased concentration of soluble calcium, magnesium and inorganic phosphate; this was taken as evidence that heating caused no permanent change in the structure of CCP.

The fate of the calcium and inorganic phosphate which become insoluble during heating of milk is not certain. From the data of Pouliot *et al.* (1989a), it was noted by Holt (1995) that in milk the concentrations of non-ultrafilterable calcium and inorganic

phosphate increased with temperature, but the ratio of calcium to inorganic phosphate in the UF retentate was constant at a value of 1.12; this ratio was similar to that of calcium and phosphate in the indigenous CCP, suggesting that the effect of increased temperature may have been to increase the size of the CCP nanoclusters in the casein micelle. Another possibility is that calcium phosphate would be deposited onto the surface of the casein micelles, constituting a separate phase from the indigenous CCP; in this case the precipitate would probably consist of dicalcium phosphate ($CaHPO_4$) or tricalcium phosphate (($Ca_3(PO_4)_2$), as speculated by (Singh and Creamer, 1992), or possibly hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), as connoted by Fox (1982). It was found by Dalgleish *et al.* (1987) that when milk was heated in a stainless steel heating system, for up to 1 h at 130°C, the [Ca^{2+}] and the [PO_4^{3-}] in sedimentable material remained constant and, at the same time, extensive precipitation of calcium phosphate occurred on the walls of the heating system, which may indicate that the precipitated minerals tend to fall out of solution, rather than associate with the indigenous CCP of casein micelles or as a separate phase onto the surface of the micelles.

Regardless of the possible fate of calcium and inorganic phosphate which become precipitated upon heating, it is generally accepted that such a reaction is accompanied by the release of H^+ ions, contributing to heat-induced pH decline of milk and also adversely affecting the heat stability of milk; these effects will be discussed in sections 4.3 and 4.4.

Dalgleish *et al.* (1987) showed that while heating caused extensive dephosphorylation of the caseins, it appeared that the released phosphate was not liberated into the serum phase of milk. These authors suggested that the structure of indigenous CCP was not altered by dephosphorylation, the effect of which was merely to convert organic phosphate into inorganic phosphate. The heat-induced dephosphorylation of the casein in milk may have an important effect on its heat stability (see section 4.2).

As discussed above, heating may or may not affect the size of CCP nanoclusters; in either case the question arises as to whether the stochiometry of the indigenous CCP is altered by heating. Lucey *et al.* (1993b) showed that when milk was heated at 120°C for 10 min, its maximum buffering index, upon acidification to pH 2, was shifted from pH 5.09 to pH 4.46; this was taken as evidence of a change in the structure of CCP, the nature of which was not established. It was observed by van Dijk and Hersevoort (1992), that when milk was heated at 128°C for 5 min, it became increasingly difficult to solubilize

the CCP by the addition of NaCl, Na_3Citrate or EDTA, which could also be interpreted as a change in the nature of the indigenous CCP. However, Holt (1995) argued that the results of most studies imply that precipitation of a thermodynamically more stable phase of CCP does not occur during heating. This author suggested that experiments which had shown a reduced rate of exchange of calcium ions between the CCP and the serum upon heating (the results of which were interpreted by some workers as evidence for a change in the stochiometry of CCP), may have been caused in reality by a change at the phase boundary or in the proteinaceous matrix of the micelle. Nelson *et al.* (1989) used X-ray absorption spectroscopy to determine the short range structure of the calcium and phosphate ions of CCP and could detect no significant change in structure, even when the CCP was heated at 120°C for 45 min.

2.3.3 Concentration processes

The concentration of milk by evaporation has a substantial effect on the milk salt system. Because less water is available for solvation of milk salts, a shift occurs from the soluble to the colloidal phase. When the concentration of total solids of milk was increased by a factor of 3.5, the soluble calcium content increased by a factor of 2 (Vujicic and Deman, 1966), indicating a shift to the colloidal state. Similar effects were reported by Pouliot and Boulet (1995) who showed that the propensity to shift from the soluble to the colloidal state, upon concentration of milk to ~ 31% total solids, was in the order $Ca^{2+} > PO_4^{3-} > Mg^{2+} >$ Citrate. Evaporative concentration of milk also leads to a decline in pH; if the concentration of skim milk is increased from 9 to 26 to 40% total solids, then the pH decreases from 6.6 to 6.2 to 6.0, respectively (Fox, 1982). Part of the pH drop is due to the increased concentration of organic acids in the milk, but the increased concentration of colloidal milk salts probably also contributes, due to the precipitation of secondary and tertiary calcium phosphates with release of H^+ ions.

As in the case of heating, it is not known if the conversion of soluble milk salts to the colloidal state involves precipitation of the salts onto the CCP nanoclusters, the surface of the casein micelle or their precipitation out of solution. Pouliot and Boulet (1995) reported that concentration of milk to 31% total solids altered the ratio of Ca^{2+} to PO_4^{3-}, that existed in the colloidal state, from ~ 1.4 in unconcentrated milk to ~ 1.9 in concentrated milk. These authors suggested that the structure of indigenous CCP was therefore altered

by concentration, but it must be pointed out that an altered ratio does not indicate where the extra colloidal milk salts actually precipitated.

Assuming that UF membranes do not exhibit differential permeabilities to low MW species, including lactose and the soluble milk salts, and that UF is carried out at a low pressure, an UF permeate of milk should have an identical composition to that of the aqueous phase of milk, less the protein and fat. Therefore, an UF retentate should contain an increased concentration of protein and CCP to soluble salt, but the composition of the soluble salts should be the same as in normal milk; this seems to have been confirmed experimentally (Green *et al.*, 1984). Indeed, UF has been used extensively in studies on the milk salt equilibria because it is supposed to cause minimal distortion of the natural salt equilibria (Holt, 1985). However, during UF the 'Donnan potential' would be expected to cause a small distortion of the true milk salt equilibria (Hiddink *et al.*, 1978; Holt, 1985). The Donnan potential arises because if an ion is present which cannot permeate an UF membrane, it may affect the permeation rate of those ions which do. In milk, the casein micelle and possibly some whey proteins, behave as if they were large, multivalent 'ions'. At natural pH, casein micelles have a net negative charge, due mainly to the negative groups of the 'hairs' of κ-casein. This results in an increased concentration of cations in the soluble phase in the vicinity of the micelle surface, causing the ultrafilterability of the cations to be reduced. Therefore, a milk UF permeate would be expected to contain slightly less cations and to show a higher pH than that of the true soluble phase of milk; it has been calculated that milk UF permeate would contain ~ 1% less monovalent or 2% less divalent cations and have a pH value ~ 0.02 units higher (Holt, 1985).

2.3.4 Additives

Due to the calcium-sequestering ability of EDTA, the addition of this compound to milk leads to solubilization of the caseins. Probably, ETDA binds to soluble calcium, thereby causing dissolution of the calcium of the CCP to restore equilibrium. The disintegration of CCP would be expected to lead to dissociation of the caseins from the micelles. When casein micelles, dissolved in milk ultrafiltrate, were dialyzed against solutions containing increasing amounts of EDTA, a progressive increase in the concentration of soluble casein occurred; at 120 mM EDTA, the casein micelles completely disintegrated, as detected by light scattering and sedimentation techniques (Lin *et al.*, 1971; Bloomfield and Morr,

1973). Conversely, it has been found that dialysis of casein micelles dispersed in milk ultrafiltrate, against a $CaCl_2$ solution led to increased association of caseins with the micelles (Lin *et al.*, 1971; Bloomfield and Morr, 1973). Using ^{1}H-nuclear magnetic resonance spectroscopy, Rollema and Brinkhuis (1989) observed that the depletion of calcium by EDTA led to an increased motility of the caseins that remained in the micelle, whereas upon acidification the caseins which remained in the micelle maintained structural rigidity. This suggested that the structure of casein micelles was altered more drastically by the presence of EDTA than by acidification.

During the manufacture of sterilized, evaporated milk, it is customary to add various kinds of salt to the concentrated milk, after its preheating, to increase heat stability. These include Na_3PO_4, Na_2HPO_4, NaH_2PO_4, $NaHCO_3$, Na_3citrate or $CaCl_2$. Determination of the kind of salt that will optimize the heat stability of the concentrated milk is largely a matter of trial and error, attesting to the fact that the mode of action of these salts is poorly understood. It is probable that the ability of these salts to stabilize concentrated milk to heat is related to a buffering action; Na_3PO_4, Na_2HPO_4, $NaHCO_3$ or Na_3citrate are added if the concentrated milk is too acidic, while NaH_2PO_4 or $CaCl_2$ are used if it is too basic (Fox, 1982). It is also possible that the addition of these compounds to milk alters the salt equilibria of milk, especially during heating, though very little is known of the nature of this change and whether it would affect heat stability. The addition of the sodium phosphate salts to concentrated milk would be expected to reduce the aCa^{2+}, because of the presence of extra HPO_4^{2-} and $H_2PO_4^-$ in solution, which would probably contribute to greater heat stability.

2.4 The freezing point of milk and its relationship to the milk salt system

It is a routine practice to measure the freezing point (FP) of milk of individual farmers in order to detect the possible presence of added water. Normal milk freezes at a temperature of about - 0.522°C, while a FP > - 0.508°C is considered to be irrefutable proof of the presence of added water (AOAC, 1990). Despite slight variations in the FP of milk, caused by regional, seasonal and other factors, FP values between individual milk samples show very little variability. This reflects the facts that milk and blood have identical molalities (moles solute L^{-1} solvent); that the FP depression is proportional to molality; and that the molality of blood hardly varies between individual cows or breeds of cow. The

relationship between FP and molality is described hereinafter, from which it should be evident that the soluble salt content of milk has a strong impact on its FP and hence, in principle, FP measurements could be used to monitor changes in milk salt equilibria.

2.4.1 General influence of solutes on freezing point

At one atmosphere, 'pure'[2] water freezes at 0°C. Above 0°C, water molecules continually associate and dissociate to form transient, ice-like structures. The formation of these structures involves the aggregation of water molecules which are attracted to one another by hydrogen bonds. The lifetime of these structures is probably in the order of picoseconds because they are continually being bombarded with water molecules from the bulk phase causing their dissipation. When the temperature of water is reduced the life time of the ice-like structures increases, because the strength of intermolecular hydrogen bonding increases and the kinetic energy of water molecules in the bulk phase is reduced. At a critical temperature (0°C), water molecules in the bulk phase can approach the ice-like clusters relatively slowly and if they strike at a suitable location and are correctly orientated can attach permanently to the ice-like structures, thus initiating ice nucleation.

The presence of solutes in water causes the FP to be depressed. The ability of a solute to depress the FP of water is likely to be related to a series of factors, including its concentration, solubility, distribution and valency of charged groups, amphipathicity, its spatial geometry, diffusivity and tendency to interact with itself and other solute species; apparently, no theory has been developed which considers the effect of all of these factors on FP. Despite the possible theoretical complexity of a true explication of the effect of a solute on FP, it has been shown empirically that, for dilute solutions, a linear relationship exists between the molality of a solute and its contribution to FP depression:

$$\text{FP depression (°C)} = C.n.M,$$

where C is a constant, M is the moles of solute kg^{-1} of solvent and n is the number of soluble species that originate from the dissociation of 1 molecule of solute. This

[2] Theoretically, absolutely pure, undisturbed water can be supercooled to about - 48°C before the spontaneous onset of ice-nucleation; this is known as homogenous nucleation. This has been verified experimentally, where it has been possible to supercool water droplets to about - 45°C. In most situations, however, even 'pure' water contains trace quantities of impurities which act as nucleation sites or the water is mechanically disturbed during cooling, enabling it to freeze at 0°C.

relationship is a variant of Raoult's Law, which states that the vapour pressure depressing effect of a solute is proportional to its molality. A value of 1.86 for C, is good for predicting the effects of a wide variety of low MW solute types on the FP of aqueous solutions, though, in principle, and sometimes in practice, different solute species have different values for C (Jenness *et al.*, 1974). From the above equation, it can be seen that the ability of a solute to depress FP depends fundamentally on the ratio of the number of solute particles to the number of solvent molecules. It follows that solutes with a high MW, for example proteins, will have little effect on FP, because even if the concentration is high, the number of molecules will be relatively low, while the opposite is true of low MW solutes, like simple sugars or especially salts.

It appears that no satisfactory explanation has been offered as to why the FP depressing ability of a solute is so fundamentally related to its molality in solution. It is possible that solute molecules interfere with ice nucleation by 'binding' to water molecules and increasing their 'residence time'[3], thus making them less available for collision and possible hydrogen bonding at potential ice nucleation sites. In this context, one could imagine that if a large particle was broken up into numerous tiny particles (for example, the hydrolysis of a protein into its amino acid constituents), the surface area of solute exposed to solvent would increase enormously and thus magnify its FP depression effect, even though the mass of solute would not change. However, the ability of a solute molecule to 'immobilize' water would be expected to depend on its polarity and number, distribution and type of charged groups present, and such effects are difficult to quantify. It is also conceivable that the much greater diffusivities of small particles would also contribute to a greater FP depression effect, as their greater motility might be expected to disturb the formation of a greater number of potential ice nucleation sites.

2.4.2 Measurement of freezing point

Measurement of FP can be carried out quickly and with remarkable precision (in the order of m°C) by the use of a cryoscope, which is used routinely by the dairy industry (AOAC, 1990). The method involves the supercooling of milk to about - 3°C, in which it exists in a

[3] The residence time of a water molecule refers to the amount of time it spends at a cetain, tiny element of volume. In the bulk phase a water molecule has a residence time in the order of picoseconds, which is increased ~ 1000 fold to the order of nanoseconds when it is 'attached' to a solute molecule. Although the term 'bound' water is used rather liberaly throughout the literature, in most cases the water molecules still exhibit very high motilities and thus this term is unbefitting.

metastable liquid state. The supercooled liquid is then subjected to a sudden vibration by a mechanical probe, which initiates rapid ice nucleation and crystallization. During ice crystallization, hydrogen bond formation leads to the conversion of chemical energy into heat energy, which causes the temperature of the sample to rise rapidly and then stabilize at the FP value. Extremely stable readings are obtained for the FP, because when the sample reaches the FP value, the temperature cannot increase any further because any extra heat energy is dissipated as the latent heat of melting of ice. In the case of milk, or indeed any aqueous solution, the FP will always be less that zero and, at and below the FP, only a certain fraction of the total water present will be in the solid state; in general, a higher solute content leads to a bigger depression of FP and a greater amount of unfrozen water.

2.4.3 Influence of solutes on freezing point of milk

Milk contains an enormous number of species dissolved or dispersed in the aqueous phase, including the numerous protein types, lactose and other sugars, soluble and colloidal milk salts, free fatty acids and fat globules. Of all these constituents, lactose and soluble salts (especially sodium, potassium and chloride, which, on a molal basis, are the most abundant soluble milk salts) by virtue of their low MW's and relatively high concentrations in milk, have by far the greatest effect on FP (van der Have *et al.*, 1980; Koops *et al.*, 1989; Mitchell, 1989). From the soluble salt composition data of Table II-5, and assuming that a typical milk contains 4.5% (w/w) lactose, it can be calculated that dissolved salts would cause the FP of milk to be depressed by ~ 0.26°C, while lactose would cause a FP depression of ~ 0.25°C, which together would cause the FP to be about - 0.51°C, which is close to the FP value of normal milk.

Other milk constituents have a much smaller effect on FP. Proteins interact with water in an extremely complicated manner via charged, polar and hydrophobic groups (Edsall and McKenzie, 1983) but due to their high MW's (~ 14 - 1000 kDa) have very low molalities in milk and hence have a finite but negligible effect on FP. This would be especially true of the casein micelles, if the micelles are considered to be discrete entities, as a typical micelle has a MW of ~ 10^8 Da. It should be noted that casein micelles and to a lesser extent whey proteins are hydrated and therefore reduce the amount of water in milk available for solvation of other species, thus indirectly contributing to a lower FP. It can be calculated that if the water of micelle hydration was reduced to zero (this might be achieved

if the micelles were subjected to extremely high centrifugal forces, causing them to sediment and express water), then the FP of milk would increase by ~ 0.037°C or ~ 7%. Assuming that the colloidal salts of the casein micelles are tightly bound, their impact on the FP would be negligible, as they would exist as part of the high MW casein micelle; in fact the existence of CCP as crystalline dicalcium phosphate indicates that CCP is not truly soluble in milk and therefore does not contribute to the FP depression. The binding of calcium to α-la or iron to lactoferrin would be expected to negate the effect of these metal ions on the FP. Most of the milk fat occurs as triglycerides, which despite their relatively low MW's have little effect on FP. This is probably because of the existence of milk fat as fat globules, which would reduce vastly the surface area of fat exposed to the aqueous phase of milk.

2.4.4 Potential use of freezing point measurement to assess changes in milk salt equilibria

The CCP can be expected to have a negligible impact on the FP of milk, whereas soluble salts contribute to about one half of the FP depression of milk. In preceding sections, it was seen that the partitioning of milk salts is affected by a number of factors, including pH, temperature, concentration and additives. Therefore, in principle the extent of changes in milk salt equilibria could be determined by measuring the FP of milk, but apparently this technique has not been attempted.

FP measurements can be made rapidly and with great sensitivity. Furthermore, FP measurements could be used to assess the state of the milk salt system within milk itself. Other techniques such as UF or dialysis, rely on the physical separation of the milk salts into their colloidal and soluble phases, and quantification of the types of salt species in each phase. A problem with these techniques is that they can distort the salt equilibria of milk; Holt (1985) noted that the composition of a milk diffusate or ultrafiltrate would not be exactly equivalent to that of the soluble milk salt system because of the 'Donnan potential' and the excluded volume effect caused by the absence of casein micelles. Other methods of evaluating the state of the milk salt equilibria involve the use of ion specific electrodes to measure the activities of certain ions, such as Ca^{2+}, Na^{+}, K^{+} or Cl^{-}. While such measurements can be made in milk itself, only a limited range of electrode types is available and the electrodes require careful calibration in accordance with temperature.

3 Influence of heating on the milk proteins*

Almost all dairy processes involve the use of heat treatment, making knowledge on the heat stability of milk and milk proteins highly desirable. The caseins and whey proteins have fundamentally different structures, which manifest themselves as contrasting physico-chemical properties, including that of heat stability: in general, the whey proteins are sensitive to heat denaturation and aggregation, whereas the caseins are not. These effects have been amply demonstrated in both model systems containing isolated whey or casein proteins and in milk and other dairy products. The heterogeneity of the milk protein system, and indeed the presence of non-protein constituents, especially lactose and salts, which may be modified by heating, make interpretation of heat stability data for milk and other dairy products difficult; despite numerous studies the heat stability phenomena of milk and other dairy products are still not fully understood. Even in the case of model systems, where the heat denaturation of isolated milk proteins has been studied, there is considerable variability between different experimental studies.

In the following sections, the general effects of heating on proteins are described, in terms of physico-chemical changes and thermodynamic and kinetic parameters, followed by descriptions of the various methodologies used to measure protein denaturation, and the denaturation of specific proteins in milk and model systems. The discussion shall be oriented mostly towards the denaturation of the globular whey proteins, because of the extensive data available and the need to make such data more succinct. Although the casein proteins have a very important effect on the heat stability of milk and other dairy products, which is modulated by the presence of whey proteins (see section 4.1), in isolated systems they appear not to exhibit thermodenaturation properties probably because of the absence of secondary and tertiary structures (see section 3.5).

3.1 General influence of heating on proteins

The tertiary structure of a globular protein refers to its detailed three-dimensional configuration due to the complex folding of a polypeptide chain. When a sequence of amino acids is synthesized, it has a spontaneous tendency to fold into a compact, globular structure in order to minimize its conformational free energy and such folding is stabilized

* A version of this section was published: Jelen, P. & Rattray, W. (1995). In: *Heat-Induced Changes in Milk.* Fox, P.F. (ed.). Special Issue No. 9501, International Dairy Federation, pp 66- 85

by hydrogen, hydrophobic, van der Waals, electrostatic and thiol-group interactions (Creighton, 1990). The conformation of a protein is susceptible to alteration by heating; Figure II-4 shows the general effect of heat on a solution of a globular protein.

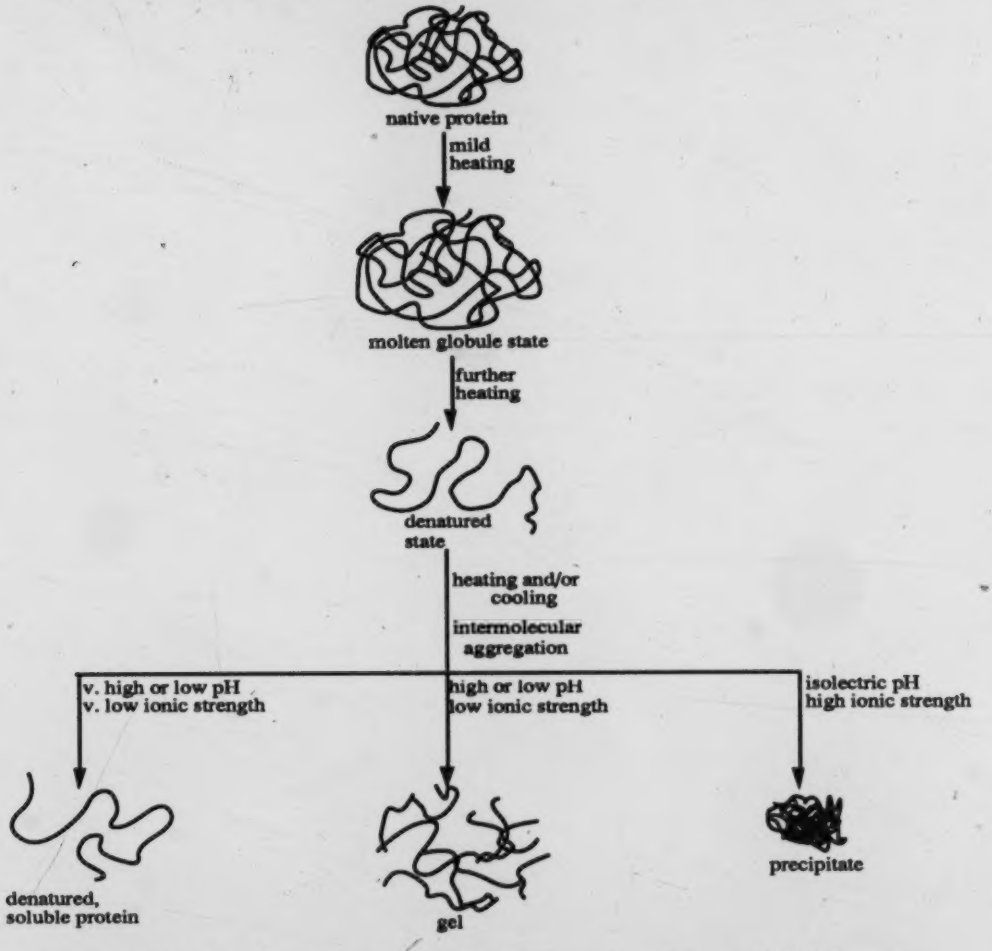

Figure II-4. Highly schematic and generalized illustration of the typical changes that occur upon heating of a solution of globular protein, as affected by pH and ionic strength.

Very mild heating of a protein solution, which typically corresponds to a temperature increase of ~ 10°C above the physiological temperature of the protein, causes the protein to be converted to the 'molten globule state'; the term 'globule' indicates that the globular structure is preserved, while 'molten' indicates increased conformational flexibility. Thus the molten globule state corresponds to a loosening of tertiary structure, accompanied by an increased voluminosity of the protein and increased contact between

solvent and the amino acid backbone. The molten globule state is distinct from the native state but does not correspond to a denatured state, because secondary structures and the overall shape of the protein are preserved. The existence of a protein in the molten globule state has a significant effect on its physico-chemical properties; for further information on this 'hot topic' see Dill and Shortle (1991) and Chyan *et al.* (1993).

Protein denaturation always involves a substantial alteration of secondary and tertiary structures, possibly accompanied by intramolecular sulphydryl-disulphide interchange reactions, but without cleavage of covalent bonds in the peptide backbone (Tanford, 1968). Denaturation of a protein usually causes it to assume a more open, much less ordered conformation. Depending on environmental conditions, especially pH and ionic strength, denatured protein molecules may undergo intermolecular association, leading to the formation of a precipitate or a gel, or at extreme pH values and low ionic strength remain soluble, due to very limited or possibly complete inhibition of aggregation. In Figure II-4, the influence of protein concentration on protein aggregation is not shown, but in many cases (though not always) a low protein concentration is conducive to the formation of a precipitate or denatured soluble protein, while a higher protein concentration is more likely to result in the formation of a gel.

3.2 Kinetic and thermodynamic parameters used to characterize the heat denaturation of proteins

The thermal denaturation of a protein may be considered to be a unimolecular reaction[4], in which the highly ordered globular conformation is disrupted to form a more extended and disordered structure. Protein denaturation is a complex process, involving the formation of numerous, metastable transition states, as the temperature of a protein solution is increased (Lapanje, 1978). To simplify matters, it is useful to consider protein

[4] Strictly speaking, protein denaturation is not a unimolecular reaction; heating increases the collision frequency between bulk water molecules and protein molecules and the transfer of momenta of the water molecules to the protein is largely responsible for the unfolding of the protein. Therefore, protein denaturation is a multimolecular reaction and it should not be surprising that no theory exists to describe, what, in principle, must be an astoundingly complex process. To simplify the situation, it is useful to consider the unfolding of a polypeptide chain in isolation, enabling the visualization of a unimolecular reaction.

denaturation as a change from the native state to a single denatured 'state'[5] (P_N--->P_D). The rate of protein denaturation equals the rate of loss of native protein, which can be stated in terms of a rate equation:

$$\frac{-d[P_N]}{dt} = k[P_N]^n,$$

where the square brackets indicate concentration, t is time, k is the rate constant and n is the order of reaction. Both k and n are intrinsic characteristics of a specific type of protein, characterizing protein denaturation as it proceeds. The value of k can be regarded as a measure of the inherent tendency of the protein to unfold at a certain temperature. The order of reaction, n, indicates how reaction rate is related to reactant concentration, which may also depend on temperature, though to a lesser extent than k. The usual method of determining n and k, for protein denaturation, involves heating a protein solution at a certain temperature for various time intervals, followed by cooling and quantification of the amount of native protein. The dependence of $[P_N]$ on time, which can be obtained by integration of the appropriate rate equation, is related to the values of n and k; for details of calculation procedure see van Boekel and Walstra (1995).

Thermodynamic parameters useful in characterizing protein denaturation include the lowest temperature at which denaturation occurs (T_D), the activation energy (ΔE^*), enthalpy (ΔH_D), entropy (ΔS_D) and free energy of denaturation (ΔG_D). The value of T_D is governed by the stability of the most heat-labile intramolecular bonds of the protein, the disruption of which initiates denaturation. The T_D should not be considered as a measure of true protein stability; in order for a protein to become totally denatured, the entire complement of intramolecular bonds must be broken; the energy required to do this, which would be a measure of total protein stability, would not necessarily be correlated to the energy needed to break the most heat-labile bonds.

The other thermodynamic parameters, namely ΔE^*, ΔH_D, ΔS_D and ΔG_D describe certain thermodynamic differences between the protein in its native and denatured states. In order to acquire an understanding of the significance of these variables, a brief divergence

[5] In reality, when a protein solution is heated to a certain temperature to cause protein denaturation, numerous conformational states are formed, those with the lowest free energies being the most populous; characterization of a single denatured 'state' corresponds to taking the average properties of a population of states. For futher information see Lapanje (1978) and Dill and Shortle (1991).

to the transition state theory of chemical reactions is necessary; for a more detailed description of this theory see van Boekel and Walstra (1995). Initially, this theory shall be described on the basis of a simple bimolecular reaction, before extending it to the case of protein denaturation.

The essence of the transition state theory is that a chemical reaction proceeds via the conversion of reactants (A + B) to an high energy, unstable intermediate entity (I*), which then decomposes into products (C, D):

$$A + B \Leftrightarrow I^* \Leftrightarrow C + D$$

It must be emphasized that the intermediate is so unstable that it exists only for a fleeting instant and its properties can never be measured directly. In this case, ΔE^* represents the minimum total energy that reactants (A, B) must have in order to be converted to the transition state (I*), while $\Delta H^{\neq}$ indicates the enthalpy difference between the reactants and the transition state. The value of ΔE^* is only slightly greater than that of $\Delta H^{\neq}$ because $\Delta E^* = \Delta H^{\neq} + RT \approx \Delta H^{\neq}$, where R is the gas constant and T is temperature. From the transition state theory, the terms $\Delta S^{\neq}$ and $\Delta G^{\neq}$ are derived, which indicate the entropy and free energy of activation, respectively. The $\Delta S^{\neq}$ can be regarded as a measure of the difference in randomness between the chemical reactants and the intermediate, which would almost invariably be large and positive. The $\Delta G^{\neq}$ is usually positive, indicating that the formation of an intermediate state is not a spontaneous process, unless the system acquires energy. The terms ΔH_R, ΔS_R and ΔG_R denote certain thermodynamic properties which differ between the reactants (A, B) and the end products (C, D). Whether these terms are positive or negative depends very much on the nature of the reaction

Protein denaturation may be regarded as a unimolecular reaction, involving the formation of numerous intermediate states between the native protein and the final denatured state. However, none of these intermediates can be regarded as being of abnormally high energy and instability. Therefore, the variables $\Delta H^{\neq}$, $\Delta S^{\neq}$ and $\Delta G^{\neq}$ are not really applicable to the case of protein denaturation and the process is merely characterized by the terms ΔE^*, ΔH_D, ΔS_D and ΔG_D, indicating a change between the native and a denatured state. Because the thermodenaturation of a protein requires the input of heat energy, ΔH_D will always be positive. The relationship between ΔE^* and ΔH_D is given by $\Delta E^* = \Delta H_D + RT \approx \Delta H_D$. The ΔH_D represents the energy input needed to convert a protein from the native to the denatured state and is related to the extent of secondary structure of

the protein, particularly the α-helix content. It follows that ΔH_D depends on the extent of secondary structures within a protein, the strength of the bonds involved in the maintenance of secondary structures and the size of the protein. The ΔS_D is almost invariably positive for protein denaturation, due to the vastly larger number of conformational states a protein can assume when it is denatured. The larger the value of ΔS_D, the more drastic is the difference between the protein in its native and denatured states. In general ΔG_D, is positive indicating that, at physiological temperature, protein denaturation is a non-spontaneous process and therefore requires the input of heat energy.

The relationship between the rate constant, k, and temperature is given by the Arrhenius equation:

$$k = k_0.\exp(-\Delta E^*/RT),$$

where k_0 is the pre-exponential factor. Thus, by plotting ln k against 1/T, it is possible to determine k_0 and ΔE^*. According to the Eyring equation, the value of k_o is related to ΔS_D, the value of which can be used to obtain ΔH_D and ΔG_D; for a detailed description of the calculation of these parameters see van Boekel and Walstra (1995). The value of ΔH_D can also be determined by the use of differential scanning calorimetry (see section 3.3.1).

3.3 Methods used to measure the thermal denaturation of whey proteins

A wide variety of methods are available to determine the kinetic and thermodynamic parameters which characterize protein denaturation (Lapanje, 1978). Thermal denaturation of globular proteins is typically accompanied by several physical, chemical and physico-chemical changes. Changes in enthalpy, solubility, spectral properties and antigenic activity have all been used to monitor whey protein denaturation.

3.3.1 Differential scanning calorimetry

The thermal unfolding of globular proteins requires the uptake of heat energy, needed primarily to rupture intramolecular hydrogen bonds (Privalov and Khenchinashvili, 1974). This endothermic heat effect can be monitored using differential scanning calorimetry (DSC); for a more rigorous discussion of this technique see Wright (1982), Paulsson (1990) and Ma and Harwalkar (1991). Using DSC a test sample, consisting of a solution of native protein, and a reference sample are subjected simultaneously to the same heating

regime. The reference may consist of same solvent used as in the test sample (usually water) or an identical solution to that of the test solution, except that the protein was heat denatured previously under the same conditions; the latter approach should not always be recommended because if the protein in the reference solution renatured, then values obtained for the enthalpy of protein denaturation would be too low. A typical DSC thermogram representing the endothermic unfolding of a globular protein is shown in Figure II-5.

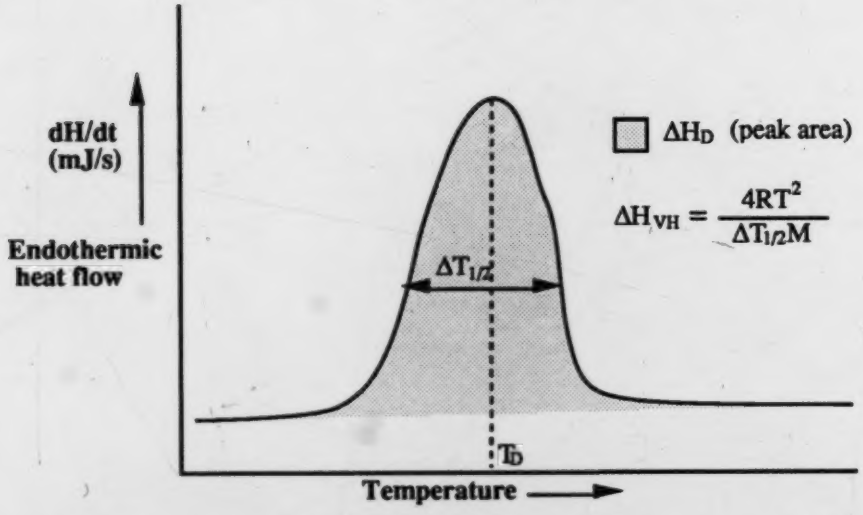

Figure II-5. A typical DSC thermogram representing the heat denaturation of a solution of globular protein.

A DSC thermogram can be used to estimate a number of criteria, the most useful of which are probably the temperature of protein denaturation (T_D), the enthalpy (ΔH_D) and the van't Hoff enthalpy (ΔH_{VH}) of denaturation. The temperature of maximum deflection of the endothermic curve is commonly denoted as the T_D, though strictly speaking this would not correspond to the temperature at which the most heat-labile bonds were broken (the definition of T_D above). The ΔH_D corresponds to the area under the denaturation curve. The ΔH_{VH} is related to the sharpness of the endothermic peak (defined as $\Delta T_{1/2}$; peak width at 1/2 peak height), and is an index of the co-operative nature of the transition from native to denatured state. More specifically, the ratio of $\Delta H_D/\Delta H_{VH}$ indicates the cooperativity of the denaturation process: when it is close to 1, the population of intermediate states

between native and denatured states is low and denaturation is likely to resemble an 'all or nothing' process; when the ratio is less than 1, denaturation is even more discrete and more likely to be irreversible; high values of $\Delta H_D/\Delta H_{VH}$ indicate that denaturation is complex and characterized by the presence of numerous intermediate states.

By using DSC the heat denaturation of proteins can be monitored directly, continuously and sensitively. Thermal characteristics can be obtained relatively quickly and because small sample sizes (the order of mg) are required, it is possible to assay a large number of samples under a variety of conditions. The technique can be used also to estimate the degree of reversibility of protein denaturation. This has been accomplished by cooling a sample of denatured protein and reheating it at the same rate (Rüegg *et al.*, 1977; Bernal and Jelen, 1984). Unless the protein is denatured irreversibly, a second peak results on reheating, the size of which is proportional to the percent renaturation. One disadvantage of DSC is the necessity to use relatively high protein concentrations; thus the method is not suitable for studies of denaturation behaviour in typical dairy systems. Another problem is that calorimetric characteristics, particularly T_D, may change as a function of the heating rate applied; if a protein is subjected to very fast heating then the value of T_D will be higher. To obtain a more objective value for the T_D, Rüegg *et al.* (1977) measured the temperature of denaturation for various whey proteins at different heating rates and extrapolated the data to obtain T_D at a heating rate of 0°C min^{-1}.

3.3.2 Solubility

The thermal unfolding of globular proteins tends to enhance intermolecular interactions by exposure of more amino acid residues, especially those which are hydrophobic, to the aqueous environment. This effect frequently leads to a loss of protein solubility which can be measured to assess the extent of whey protein denaturation (Larson and Rolleri, 1955; Harwalkar, 1979, 1980a; Harwalkar and Kalab, 1985; Patocka *et al.*, 1986).

Solubility measurements are almost invariably carried out after cooling of the heated protein solution, and therefore only irreversible denaturation can be detected. Furthermore, denaturation is not always accompanied by a loss of solubility, especially at pH values removed from the isoelectric point. For many years, α-la was believed to be the most heat stable whey protein, because it maintained a high solubility upon heating; however, using DSC, a number of studies have shown that the protein unfolds at a relatively low

temperature, yet remains highly soluble, even at high ionic strength. Harwalkar (1979) observed that whey proteins remained highly soluble following extensive heating (90°C for 30 min) at pH 2.5, but were readily precipitated at pH 4.6; these observations were confirmed by Patocka *et al.* (1986). It was reported by de Wit (1981) that solutions of β-lg remained almost crystal clear after heat treatment (80°C for 10 min) at ≥ pH 6.8, but below ~ pH 6.5, there was a dramatic increase in the amount of protein precipitated. Bernal and Jelen (1985) observed that there was no loss of solubility of the total whey protein fraction, when acid whey was heated at 95°C/5 min, at < pH 3.7, but at > pH 3.9, heating led to protein coagulation.

To overcome these difficulties, many studies have been carried out by heating a solution of milk protein at a certain pH and precipitating the denatured proteins at pH 4.6. The precipitated coagulum can be centrifuged and the supernatant obtained is assumed to contain the native proteins; total native protein can be quantified by Kjeldahl or individual proteins by polyacrylamide gel electrophoresis (Hillier, 1976; Hillier and Lyster, 1979; Hillier *et al.*, 1979), high pressure liquid chromatography (Parris and Baginski, 1991) or gel permeation fast protein liquid chromatography (Law *et al.*, 1993).

3.3.3 Immunology

Whey proteins are antigenic and therefore small structural changes can affect their ability to bind to antibodies; protein denaturation would be expected to cause a decrease in antigenic activity, which is the principle of several immunoassay techniques. Micro complement fixation (Levine and van Vunakis, 1967) involves measuring the degree of complementarity between a denatured protein and an antibody which displays maximum binding for the protein in the native state; the amount of complement (antibody) fixed at different concentrations of protein (antigen) is determined. By comparing the complement fixation percent between native and denatured proteins, the degree of protein denaturation can be inferred; greater quantities of heated proteins are needed to achieve the same complement fixation percent compared to native protein. This technique is reported to be very sensitive and applicable to both simple model systems and complex media (Baer *et al.*, 1976, 1979). By performing complement assays on solutions of whey protein, which had been heated and allowed to cool for different time periods, the ability of the proteins to renature was determined (Baer *et al.*, 1979).

Lyster (1970) used an immunodiffusion procedure to measure the thermal denaturation of α-la and β-lg in skim milk. Samples of heated skim milk were cooled, diluted and placed over a column of agar containing antiserum. After incubation at 37°C, a distinct front was obtained which was assumed to consist of native proteins complexed to antibodies. The distance migrated by the front was proportional to the concentration of native protein. Electroimmunodiffusion is a modification of immunodiffusion, but gives greater sensitivity. In this method, as used by Babajimopoulos and Mikolajcik (1978), antiserum against bovine serum albumin was allowed to adsorb uniformly onto a cellulose acetate plate. Native and denatured protein samples were applied to the edge of the plate and the plate subjected to electrophoresis with phosphate buffer, at 4°C and a current of 5 mA. After staining, a series of peaks was obtained and peak height was found to be proportional to the amount of native protein.

Techniques based on immunology can only be used to detect irreversible changes in protein conformation and, from the descriptions of methodology, it appears that these methods are tedious to execute. Moreover, there exists the possibility that even if a protein had been irreversibly denatured, its binding affinity for an antibody would not be altered; this could arise if conformational changes of the protein occurred, but were restricted to certain domains of the molecule, which did not include the antigenic epitope.

3.3.4 Spectral methods

The optical activity of whey proteins is caused by the presence of a large number of asymmetric carbon atoms, which interact with and alter certain attributes of polarized light. The interaction of polarized light with proteins can be monitored by using optical rotary dispersive (ORD) and circular dichroic (CD) spectroscopy, which have yielded valuable information on the secondary structures of proteins and their responses to heat. The interaction of polarized light with chiral centres is described by a rather abstruse theory, but a simplified description of ORD and CD was provided by Freifelder (1976) and Arnebrant and Nylander (1987). ORD and CD spectroscopy are usually carried out using light in the ultraviolet range ($\lambda \sim 175 - 340$ nm). Essentially, ORD spectroscopy involves measuring the degree of rotation of the plane of polarized light, due to the different refractivities for the left and right circularly polarized components of plane polarized light when it interacts with a chiral centre; an ORD spectrum plots specific rotation against wavelength. CD is based

on the fact that when plane polarized light interacts with an optically active molecule, the left and right circularly polarized components are absorbed to different extents, which causes the incident light to become elliptically polarized; a CD spectrum plots ellipticity against wavelength. ORD and especially CD are among the most sensitive techniques for the detection of structural changes in proteins and can measure thermodenaturation as it proceeds, without the need for sample cooling. Using ORD Harwalkar (1980a,b) and Harwalkar and Kalab (1985) monitored the thermal unfolding of β-lg and observed an increase in the levorotation of the plane of polarized light, due to increase exposure of asymmetric centres to the solvent. Matsumura *et al.* (1994) found that CD was very sensitive for detecting structural changes in α-la; at 40°C, small structural changes, probably corresponding to the transition to the molten globule state were detected.

Proteins absorb infrared (IR) light in a complicated manner; an IR spectrum consists of numerous absorption peaks superimposed on one another to generate an overall absorption spectrum; each peak corresponds to a particular vibrational transition, involving a change in the mode of vibration and/or orientation of a specific bond type. The overall absorption spectrum can be resolved into its individual peaks by second order differentiation, deconvolution and non-linear regression analysis (see Kumosinski and Farrell, 1993); this is the principle of Fourier transform infra red (FTIR) spectroscopy. The amide I absorption band of IR light, corresponding to wave numbers ~ 1600 - 1700 cm^{-1} (λ = 590 - 625 nm) is produced by various vibrational transitions involving carbonyl groups of a protein; the nature of the transitions depends on the types of hydrogen bonding that the carbonyl groups participate in and thus the kinds of secondary structures that exist. It follows that FTIR spectroscopy is a powerful technique for determining the types of secondary structures in proteins and measuring changes in these structures caused by denaturation. FTIR spectroscopy can be used to measure protein denaturation on a semi-continuous basis; in a typical experiment, Boye *et al.* (1995) heated a whey protein solution at 5°C increments and equilibrated the solution for 15 min at each temperature before performing the IR scan. A minor disadvantage of FTIR spectroscopy is that the sample must be dissolved in deuterium, as water absorps IR radiation strongly. However, Boye *et al.* (1995) and van Stokkum *et al.* (1995) used this requirement to their advantage; hydrogen-deuterium exchange between a protein and a solvent was affected by protein conformation and such could be monitored by FTIR spectroscopy, enabling the detection

of subtle conformational changes, which probably corresponded to a transition to the molten globule state.

The β-lg molecule contains two tryptophanyl residues which are normally buried in the interior of the protein. Upon heating, these residues become exposed to the aqueous environment and alter the fluorogenic behaviour of β-lg; with an excitation wavelength of 295 nm, a shift towards the fluorescence maximum (350 nm) of free tryptophan occurred with increasing protein denaturation (Mills, 1976) and similar results were reported by O' Neill and Kinsella (1988). Baer *et al.* (1979) used fluorometry to monitor the thermal denaturation and renaturation of lactoferrin and apo-lactoferrin; at an excitation wavelength of 280 nm a progressive shift towards larger emission wavelengths (up to 243 nm) occurred. The heat-induced exposure of tryptophanyl residues also affects the ability of proteins to absorb ultraviolet light. Kella and Kinsella (1988) observed an increase in the absorbance of ultraviolet (293 nm) light with increased heat treatment of β-lg solutions and a similar method was used by Kuwajima and Sugai (1978) to monitor the thermal unfolding of α-la.

3.3.5 Other methods

Jang and Swaisgood (1990) used biorecognition as an indicator of native structure in β-lg. Trans-retinal (an analogue of retinol) was immobilized on amino-propyl glass beads by reductive amidation. The glass beads were held in a column which was connected to an HPLC unit. Samples of heat-treated β-lg were applied to the column. The greater the degree of protein denaturation, the faster was the elution of β-lg, as the denatured protein had a lower binding affinity for trans-retinal.

Harwalkar (1979, 1980a) compared gel permeation chromatographs of native and heated (90°C) β-lg solutions. Heat-altered β-lg required a smaller volume of eluent and the elution profile consisted of a broad, poorly-defined peak as compared to a sharp peak for native β-lg. It was considered that heating induced limited protein aggregation and/or an increase in the effective radii of protein molecules, permitting faster elution.

Kawakami *et al.* (1992) measured the intrinsic viscosity of lactoferrin before and after heat treatment, on the basis that irreversible denaturation of a globular protein would be expected to increase the intrinsic viscosity because of a more open protein conformation.

These authors also assessed the extent of irreversible denaturation of lactoferrin by measuring its iron-binding ability, which was proportional to absorbance at 465 nm.

Anema and McKenna (1996) measured the concentration of native α-la, β-lg A and β-lg B in unheated or heated whole milk by performing gel electrophoresis in the absence of dissociating or reducing agents. The method assumed that electrophoresis would only be capable of resolving native proteins, because denaturation would lead to the formation of protein polymers which could not be separated. Despite the relatively simplicity of this method, the authors generated a large amount of thermodynamic and kinetic data. Additionally, the gel electrophoretic procedure led to separation of β-lg A from β-lg B, allowing measurement of denaturation parameters for both genetic variants in milk.

3.4 Kinetics and thermodynamics of heat denaturation of individual whey proteins

Assessment of literature reveals that conflicting data exist on the denaturation temperatures of individual whey proteins and their order of heat stability. These conflicts reflect differences not only in methodology, but also in the various conditions under which thermodenaturation took place (Harper and Zadow, 1984). For example, a literature search by Paulsson and Dejmek (1990) revealed large variations for thermodynamic data of whey proteins, as measured by DSC; experimental conditions varied widely making it difficult to assign precise values. The results of one DSC study undertaken by de Wit *et al.* (1983) are presented in Table II-7.

Table II-7. Some thermodynamic properties of the major whey proteins, as determined by differential scanning calorimetry[1].

Protein	T_D (°C)	ΔH_D (kJ mol^{-1})	ΔH_{VH} (kJ mol^{-1})[2]	$\Delta H_D / \Delta H_{VH}$
β-Lactoglobulin[3,4]	74	249	409	0.61
α-Lactalbumin[4]	63	193	256	0.75
Bovine serum albumin	87	1431	463	3.09
Immunoglobulin fraction	79	~ 3000[5]	-	-

[1]: Based on the data of de Wit *et al.* (1983), who used DSC of aqueous 10% protein solutions, adjusted to pH 6.5, heated to ~ 100°C, at a heating rate of 5 °C/min.
[2]: Tentative values calculated by Paulsson and Dejmek (1990) from the data of de Wit *et al.* (1983).
[3]: Values for β-lactoglobulin are for the first denaturation peak (~ 70 - 80°C).
[4]: Genetic variants not specified.
[5]: Value estimated for a monomeric unit of the immunoglobulin fraction.

All of these results were obtained with simple aqueous systems at the same protein concentration and pH. These results give some indication of the general heat sensitivities of the individual proteins but they are not in full agreement with results of other studies, particularly those using more complex systems.

3.4.1 β-Lactoglobulin

The thermal behaviour of whey and most whey protein products is dominated by β-lactoglobulin (β-lg), the most abundant whey protein. Although β-lg exhibits a well developed quaternary structure, little attention has been paid to the role of such in its heat denaturation; implicit in the work of most authors is that β-lg occurs as a monomer prior to its thermal unfolding. At room temperature (20°C) and pH 7.0, the protein exists in dynamic equilibrium between its dimeric and monomeric forms and very mild heating (>30°C) promotes conversion to the monomeric state. This transition is accompanied by increased solvent exposure of histidyl, tyrosinyl and tryptophanyl residues (Townend *et al.*, 1969) and by increased thiol reactivity (Dunnill and Green, 1966). At higher temperatures (> 40°C), the monomer of β-lg undergoes small, reversible conformational changes (Dupont, 1965; Kella and Kinsella, 1988), which may correspond to a transition to the molten globule state. Further heating causes extensive and irreversible protein denaturation, which is generally regarded as the classical denaturation step.

Studies involving the use of DSC (Rüegg *et al.*, 1977; de Wit and Swinkels, 1980; Hegg, 1980; de Wit and Klarenbeek, 1981; de Wit *at al.*, 1983; Park and Lund, 1984; Bernal and Jelen, 1985; Paulsson *et al.*, 1985; Paulsson and Dejmek, 1990) have indicated that β-lg denatures at 50 - 85°C, depending on environmental conditions, especially pH. Near neutral pH (~6.5 - 7.0), reported values for the T_D of β-lg are in the range ~ 75 - 80°C, while ΔH_D varies from about 136 to 368 kJ mol^{-1} protein (Paulsson and Dejmek, 1990). When the pH of simulated milk ultrafiltrate was increased from pH 3 to pH 9, the T_D of β-lg decreased from 80 to 50°C (Paulsson *et al.*, 1985); similar trends were reported by de Wit (1981), de Wit and Klarenbeek (1984), Kella and Kinsella (1988) and Boye *et al.* (1995). The decreased stability at higher pH values is partially explained by increased thiol reactivity (Watanabe and Klostermeyer, 1976). Using far ultraviolet CD spectroscopy, Matsuura and Manning (1994) detected alkaline denaturation of β-lg at

pH 10, due to the unraveling of α-helix and the formation of random coil structure; this 'pre-denaturation' would also be expected to contribute to reduced thermal stability.

Heating disrupts hydrogen and hydrophobic bonds with a consequent loss of secondary and tertiary structures and the exposure of apolar residues and thiol groups to the aqueous environment (Dupont, 1965; Timasheff *et al.*, 1967). Sawyer *et al.* (1985) have demonstrated, using ORD and ultraviolet spectroscopy, that conformational changes involve the unfolding of α-helices with attendant induction of random coil and β-structures. The low ratio of ΔH_D to ΔH_{VH} for the denaturation of β-lg (Table II-7) indicates that denaturation is a cooperative process, involving the formation of a relatively small number of intermediate states, consistent with the fact that denaturation is not completely reversible.

Using DSC, de Wit (1981) and de Wit and Klarenbeek (1981) characterized the thermal denaturation of β-lg, from pH 3.5 - 8.0, at zero ionic strength, by two endothermic peaks at ~ 70 and ~ 130°C. It was postulated that at ~ 70°C protein unfolding was accompanied by increased thiol reactivity and that intra- and intermolecular sulphydryl-disulphide interchange reactions led to the formation of an intermediate transition state which temporarily restricted further protein unfolding. At ~ 130°C disruption of the stabilizing thiol bonds occurred and a second, smaller endothermic peak was observed. At ≤ pH 6.5 or in the presence of mercaptoethanol, the 140°C peak disappeared presumably due to the inactivity of thiol groups under these conditions.

Inconsistent results exist on the kinetics of β-lg denaturation, reflecting the complexity of the process and variations in methodology and environmental conditions. Near neutral pH, Gough and Jenness (1962) and de Wit and Swinkels (1980) reported a predominantly first order process; the latter authors calculated a values for ΔE^* of 341 kJ mol^{-1}. However, in skim milk at pH 6.7, Hillier (1976) and Hillier and Lyster (1979) described the process as being second order. Park and Lund (1984) found that the order was dependent on pH; from pH 6 to 9 the process was second order, but below pH 5 it was third order; the ΔE^*, with an average value of 523 kJ mol^{-1}, was largely unaffected by pH. Harwalkar (1980b) reported that the heat denaturation of β-lg, in an aqueous solution at pH 2.5, was a pseudo first order reaction. Harwalkar (1986) observed a first order thermodenaturation process for β-lg in aqueous solution near neutral pH, but in acid whey, adjusted to pH 2.5 - 6.5, complicated kinetics were observed, which were best described as second order.

Dannenberg and Kessler (1988a-c) and Anema and McKenna (1996) described a two stage process for the thermal denaturation of β-lg in milk; both studies found that the reaction order was 1.5 for each stage. Some of the kinetic parameters calculated by Anema and McKenna are shown in Table II-8, which are in good agreement with those determined by Dannenberg (1986) and Dannenberg and Kessler (1988a-c). Dannenberg and Kessler (1988b) also observed a two stage process, when β-lg was heated in sweet whey, but each stage was second order with ΔE^* values of 331 and 74 kJ mol^{-1}.

Table II-8. Some kinetic and thermodynamic properties for denaturation of β-lactoglobulin A and α-lactalbumin in reconstituted skim milk[1].

Protein	*Heating range (°C)*	*k* $(s^{-1} \times 10^3)$[2]	ΔE^* *(kJ mol^{-1})*	ΔH_D *(kJ mol^{-1})*	ΔG_D *(kJ mol^{-1})*	ΔS_D *(kJ mol^{-1} K^{-1})*
β-Lactoglobulin A[3]	70 - 85	27	263	261	100	0.45
	100 - 115	175	51	48	100	- 0.14
α-Lactalbumin[4]	70 - 80	2	195	192	108	0.25
	85 - 115	12	58	54	108	- 0.14

[1]: Approximate values obtained from the data of Anema and McKenna (1996).
[2]: Values of k at 85 and 115°C for β-lactoglobulin A and 80 and115°C for α-lactalbumin.
[3]: Values for β-lactoglobulin B also reported by Anema and McKenna (1996), being very similar to those for β-lactoglobulin A.
[4]: Genetic variant not specified.

Of note is the fact that ΔG_D for each stage is quite large and has similar values, whereas ΔS_D for the initial stage is relatively large and positive, while for the second stage it becomes negative. This led Anema and McKenna (1996) to suggest that the initial stage of the process involved protein unfolding, as evident by an increase in entropy, whereas the second stage was due to aggregation of β-lg, which would be expected to reduce protein motility and hence entropy. This hypothesis was supported by the works of Matsuura and Manning (1994) and Boye *et al.* (1995), which showed, using CD or FTIR, respectively, that prolonged heating of β-lg led to an increase in β-sheet structure, probably due to the aggregation of β-lg into a network. Matsuura and Manning (1994) reported that when a solution of β-lg, at pH 7, in the presence of 20 mM NaCl, was heated at 90°C for 60 min it formed a gel, and far ultraviolet CD spectroscopy indicated that ~ 100% of the structure of β-lg existed as β-sheet structures.

3.4.2 α-Lactalbumin

Contrary to findings from early studies which used heat-induced aggregation as an indicator of denaturation, α-lactalbumin (α-la) is now considered to be one of the most heat sensitive milk proteins (Rüegg *et al.*, 1977; de Wit and Swinkels, 1980; de Wit and Klarenbeek, 1984; de Wit *et al.*, 1983; Bernal and Jelen, 1984, 1985). At pH 6.7, in the presence of calcium, α-la denatures at about 65°C (de Wit and Swinkels, 1980). A survey of work carried out by various authors give ΔH_D values in the range 186 - 318 kJ mol^{-1} (Paulsson and Dejmek, 1990); it is difficult to find agreement due to wide variations in experimental conditions. Despite its propensity to heat denaturation, α-la is quite resistant to heat coagulation (Larson and Rolleri, 1955; Shukla, 1973). Denaturation is 80 to 90% reversible under heating from 20 to 110°C, at pH 6.7 (Rüegg *et al.*, 1977), which probably explains the high apparent thermal stability of α-la, when denaturation is measured by a loss of protein solubility after cooling. Renaturation is assisted by the small size of the protein (123 amino acid residues) and the presence of four disulphide bonds, which restrict the number of conformational states that the protein can assume. Calcium is essential for the maintenance of the native structure of α-la and hence for its renaturation (Bernal and Jelen, 1984, 1985; Paulsson *et al.*, 1985; Patocka and Jelen, 1991b).

Calcium also has a substantial influence on the thermal transitions of α-la. At pH 7.5, α-la showed a transition temperature of 58°C, as detected by CD, but at pH 7.5, in the presence of 1 mM EDTA, the structure of α-la was altered at 32°C (Hiraoka *et al.*, 1980). Kuwajima and Sugai (1978) detected structural changes in apo-α-la at 40°C and pH 6.82, but the addition of 0.1 M Ca^{2+} shifted the transition temperature to 62°C. Similarly, using CD spectroscopy, Matsumura *et al.* (1994) detected changes in the structure of α-la, at 40°C and pH 7.5, in the presence of 5 mM EDTA, a calcium chelator. The changes in structure reported by the above workers were subtle and in some cases may have corresponded to the conversion of α-la to the molten globule state, but nevertheless point to a greater susceptibility of α-la to thermal denaturation in the absence of calcium. Bernal and Jelen (1985) reported that over the pH range 4.5 - 6.5, the T_D for α-la, as measured by DSC, was ~ 61°C, while at pH 3.5, T_D declined to ~ 58°C; the greater heat sensitivity of the protein at low pH was probably caused by dissociation of calcium with initial loss of some of its native conformation.

Studies undertaken by Lyster (1970), Hillier and Lyster (1979), Harwalkar (1986), Dannenberg (1986), Dannenberg and Kessler (1988a-c) and Anema and McKenna (1996) all indicate that, near neutral pH, the heat denaturation of α-la obeys a first order reaction; in most cases, a two stage process was observed, analogous to the situation for β-lg. Table II-8 indicates various kinetic parameters for the heat denaturation of α-la in milk, as determined by Anema and McKenna (1996).

The reversibility of α-la denaturation decreases with increasing temperature. Schnack and Klostermeyer (1980) showed that when α-la was heated at pH 7, in the range 60 - 100°C, the protein did not aggregate, but heating in the range 100 - 150°C resulted in the formation of a series of disulphide-linked oligomers, whose average size increased with the severity of heating. Likewise, Chaplin and Lyster (1986) reported that when α-la was heated in the presence of 0.1 M phosphate, at pH 7 and 100°C, the protein underwent irreversible aggregation, but the extent of aggregation was limited allowing α-la to remain soluble.

3.4.3 Bovine serum albumin

Thermal analysis of bovine serum albumin (BSA) revealed complex thermal behaviour (Rüegg *et al.*; 1977; de Wit and Klarenbeek, 1984; Bernal and Jelen, 1985; Paulsson *et al.*, 1985), probably due to the high MW of BSA and presence of numerous disulphide bonds. Sulphydryl-disulphide interchange reactions during heating are likely to be prolific permitting the formation of a multitude of intermediates (Rüegg *et al.*, 1977; Hillier and Lyster, 1979) and the non-cooperative nature of denaturation is also indicated by the high ratio of ΔH_D to ΔH_{VH} (Table II-7). Using DSC, Boye *et al.* (1996) found that the T_D for BSA was slightly dependent on pH; the peak temperature of denaturation, at pH 3, 5 or 9, was 63, 65 or 63°C, respectively. The results indicated that BSA may have been partially denatured or converted to the molten globule state at pH 3 or 9, possibly due to increased intramolecular repulsive forces. Bernal and Jelen (1985) observed a similar pattern of results and suggested that a reduced T_D of BSA at alkaline pH may have been due to increased thiol group reactivity. FTIR spectroscopy has shown that the heat denaturation of BSA is accompanied by the unraveling of α-helices, followed by the formation of non-native β-sheet structures, the latter effect being due to the initiation of aggregation rather than denaturation (Boye *et al.*, 1996).

The kinetics of the thermodenaturation of BSA have not been described satisfactorily. Hillier and Lyster (1979) found that, in skim milk, the process could be described equally well as being first or second order. Arrhenius plots showed that, like α-la and β-lg, the ΔE^* for denaturation of BSA was dependent on temperature with a much lower value at temperatures above 90°C (ΔE^* values not reported). Studies have indicated ΔH_D values of ~ 700 - 1500 kJ mol^{-1} (Paulsson and Dejmek, 1990); the high values indicate that a large amount of secondary structure is broken during denaturation, presumably a direct consequence of the large size of the BSA molecule.

The binding of fatty acids to BSA has been shown to cause a markedly increased heat stability (Gumpen *et al.*, 1979; de Wit *et al.*, 1983; Bernal and Jelen, 1985). Blood serum albumin is known to bind fatty acids in the blood; perhaps fatty acids stabilize the tertiary structure of BSA in a manner analogous to the stabilization of α-la by calcium. Also, it has been shown that NaCl, sucrose, lactose and glucose increase the heat stability of BSA (Boye *et al.*, 1996), though the mechanism is unknown. Probably, NaCl would increase heat stability by promoting intramolecular electrostatic interactions.

3.4.4 Immunoglobulins

Relatively little information can be found on the thermal denaturation of the immunoglobulin (Ig) fraction of milk. The large size, structural complexity, and the presence of numerous genetic variants, indicate that denaturation of the Ig fraction should be a very complex process. Larson and Rolleri (1955) found that the Ig fraction was the most resistant of the whey proteins to heat precipitation. A denaturation temperature of 72°C for IgG was reported by de Wit and Klarenbeek (1984) who also observed that its thermal stability was reduced at acidic pH. Rüegg *et al.* (1977) observed a single endothermic peak for γ-globulin (a class of IgG) at about 80°C when heating at 10°C min^{-1}; when extrapolated to a heating rate of zero the T_D value was 72.9°C and the ΔH_D was 4120 kJ mol^{-1}. Renaturation of γ-globulin did not occur on cooling. Law (1995) showed that, when skim milk was heated at 70 - 90°C for 0.25 - 30 min, the Ig fraction was most sensitive to irreversible denaturation, with a value of 1.5 for the order of reaction. Luf (1996) reported that the heat denaturation of the Ig fraction in milk obeyed a two stage process, a break in the Arrhenius plot occurring at ~ 75°C; the initial stage of the process

displayed a reaction order in the range 0.83 - 2.25, while the for the second stage it varied from 0.55 - 0.66.

3.4.5 Lactoferrin

The thermal behaviour of lactoferrin is strongly influenced by its ability to bind iron. Rüegg *et al.* (1977) demonstrated a single denaturation peak for apo-lactoferrin at about 65°C, but iron-saturated lactoferrin exhibited a complex denaturation thermoprofile with maximum heat uptake at 69 and 83°C. The binding of iron to lactoferrin also increased the ΔH_D from 2100 to 2900 kJ mol^{-1}. A similar trend was observed by Sanchez *et al.* (1992); in skim milk, apo-lactoferrin had an endothermic peak at 61°C with a ΔH_D of 684 kJ mol^{-1}, while iron-saturated lactoferrin exhibited two endothermic peaks at 74 and 87°C with a total ΔH_D of 1600 kJ mol^{-1}. The thermodenaturation process was first order with ΔE^* values of 158 or 203 kJ mol^{-1}, for apo- or iron-saturated lactoferrin, respectively. The stabilizing effect of iron was confirmed by Paulsson *et al.* (1993) who showed that iron-saturated lactoferrin underwent thermal transitions at 65 and 93°C, with ΔH_D values of 166 and 3071 kJ mol^{-1}, respectively, compared to apo-lactoferrin which denatured at 71 and 90°C, with respective ΔH_D values of 996 and 166 kJ mol^{-1}; the existence of two thermal transitions for apo-lactoferrin was not reported elsewhere. The heat denaturation of lactoferrin is probably irreversible. Baer *et al.* (1979) and Kawakami *et al.* (1992) reported a substantial restoration of iron binding ability on cooling but serological and fluorogenic activity were quite different from the native protein. Using DSC, Rüegg *et al.* (1977), Sanchez *et al.* (1992) and Paulsson *et al.* (1993) could not detect reversibility.

3.4.6 Other serum proteins

Information on the heat responses of other minor whey proteins is incomplete or non-existent. This includes the proteose-peptone (PP) fraction which is considered to be highly heat resistant. Three distinct PP fractions, referred to as PP components 3, 5 and 8 can be identified in highly heated whey by polyacrylamide gel electrophoresis (Jelen *et al.* 1973). The heat resistant nature of these minor proteins is probably due to their relatively small size and non globular nature. Pearce (1980) showed that when acid whey, which had been neutralized to pH 6.5, was heated at 85°C for 15 min, most of the protein coagulated, but a significant amount of soluble nitrogen remained, which probably

corresponded to the PP fraction. Similarly, Valdicelli *et al.* (1988) showed that when sweet whey was heated at up to 95°C, the PP remained highly soluble, while substantial precipitation of the other whey proteins occurred; these authors showed also that pH values in the range 5.8 - 6.4, had little effect on heat stability of PP. It is likely that from the standpoint of heat-related behaviour of the total whey protein fraction of milk or whey, the role of the PP fraction is unimportant.

Apparently, nobody has attempted to measure the heat denaturation properties of other minor whey proteins, such as folate-binding protein, vitamin B_{12}-binding protein or β_2-microglobulin, probably because it would be difficult and expensive to isolate sufficient amounts of these proteins for thermal analysis. The low amounts of these proteins in milk would be unlikely to impart a significant effect on its heat stability. However, it could be argued that if the structure and hence binding properties of these proteins were altered by heating, an effect on the nutritional quality of milk would result, for example if denaturation was accompanied by a loss of vitamin binding ability.

3.5 Influence of heating on casein proteins

Although a considerable amount of data are available on the heat stability of casein micelles in milk or in model systems (see section 4), very little work has been carried out to investigate the 'thermal denaturation' of individual casein proteins, in the isolated state or as part of the casein micelle. This probably reflects the fact that there is very little to actually report; the native structures of the caseins display extended conformations or are already 'denatured' (this is a somewhat controversial statement - see section 1.1); therefore, heating has little effect on the structures of the caseins.

In a study, the primary purpose of which was to determine the influence of casein proteins on the thermal denaturation of whey proteins, Paulsson and Dejmek (1990) also observed that heating of the α-caseins, β-casein or κ-casein from 25 - 140°C did not lead to any endothermic peak on a DSC thermogram. Using ^1H nuclear resonance spectroscopy, Lambelet *et al.* (1992) noted that heating a 12% solution of sodium caseinate or casein micelles, at 90°C for 30 min, did not produce any change in the T_2 relaxation times, indicating that changes in protein conformation did not occur. These studies suggest that all of the caseins are devoid of secondary structures and, in their native state, exhibit open conformations equivalent to being already denatured. This tends to confute the surmization

of Swaisgood (1992) that, based on spectral data, the caseins do possess significant amounts of secondary structures, but supports Sawyer and Holt (1993) who asserted the absence of secondary structures in the caseins.

4 Heat stability of milk

At its unaltered pH (~ 6.7) bovine milk can typically withstand heat treatments of up to 20 min at 140°C, before the formation of visible protein clots. The extent of this heat treatment exceeds greatly that of even the most severe heat treatments used by the dairy industry, such as ultra-high temperature heating (140°C/3 - 8 s) or in-can sterilization (110 - 120°C/5 - 10 min). Thus, the heat stability of unconcentrated milk is not an issue of practical concern. Nonetheless, small adjustments in the pH of milk (~ 0.1 - 0.5 pH units) before heating have a marked effect on heat stability and it has been a considerable challenge to elucidate the mechanisms responsible for the relationship between the heat stability of milk and its initial pH, even though pH adjustment is not used before industrial heat treatments. The plenteous literature on the heat stability of milk was the subject of reviews by Fox (1982), van Boekel *et al.* (1989), Singh and Creamer (1992) and McCrae and Muir (1995), from which it is evident that the topic is still not completely understood.

The heat stability of milk is frequently measured by the subjective heat stability test method of Davies and White (1966). A precise volume (1- 2 mL) of milk is transferred to a glass tube, the tube sealed, placed in an oil bath (120 - 140°C), rocked and heat stability taken as the time for visible protein clots to form - the heat coagulation time (HCT). On the basis of HCT/pH profile, the milk of individual cows may be classified as type A or type B (Figure II-6). Most individual cow milk samples and bulk milk are type A; over the initial pH range ~ 6.4 - 6.7, heat stability increases, a pronounced minimum occurs near initial pH 6.9, which is followed by a rapid recovery in heat stability as the initial pH is increased above 6.9. Type B milk shows a continuous increase in heat stability with increasing pH, and is less heat stabile than type A milk from ~ pH 6.4 - 6.7, but more heat stable in the region of the minimum of type A milk.

A more objective method of determining heat stability (White and Davies, 1966), involves heating milk at a certain temperature for various time intervals, followed by cooling and low speed centrifugation (300 x g) to sediment the heat-aggregated protein, which equals the difference between the total nitrogen content of the milk and the nitrogen

content in the supernatant of the heated and centrifuged milk. This method gives more detailed information on the heat stability of milk, but is more laborious to perform. For the latter reason, the subjective HCT test is the most widely used method to determine the heat stability of milk. Other methods for determining heat stability of milk include attempting to correlate ethanol stability to heat stability, measurement of viscosity or the degree of 'whiteness' in milk (Singh and Creamer, 1992), but these have received limited attention.

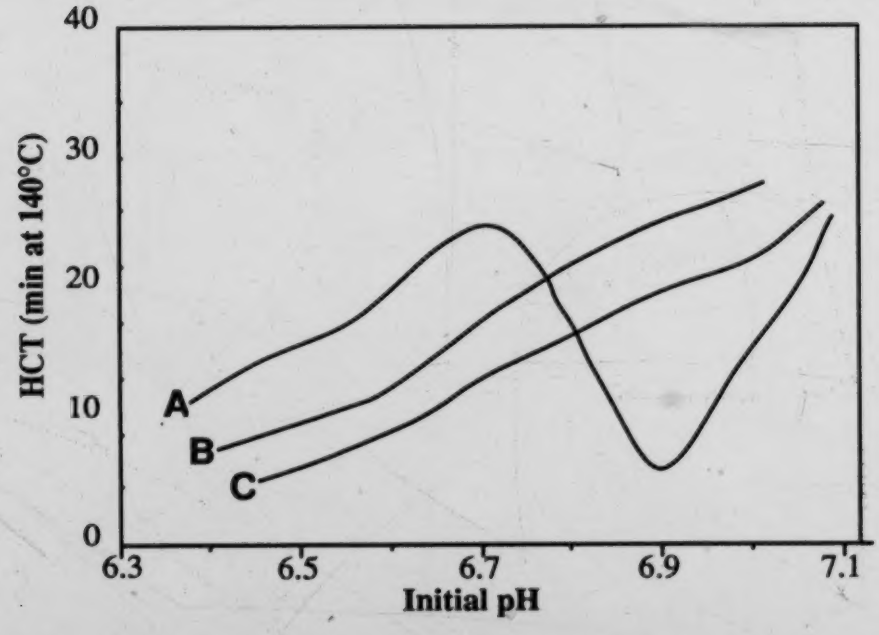

Figure II-6. Influence of initial pH on the heat stability, measured as the heat coagulation time (HCT) at 140°C, of type A milk (A), type B milk (B) or casein micelles dispersed in milk diffusate (C). Adapted from Singh and Creamer (1992).

The various physico-chemical changes which occur upon severe heating of type A milk and their relationship to the heat stability of milk are shown in Table II-9. The relevance of these changes to the thermal behaviour of type A milk shall be described in sections 4.1 - 4.6, before discussing the heat stability phenomena of type B and concentrated milk in sections 4.7 - 4.8, which are rather less well characterized.

Table II-9. Some physico-chemical changes in type A milk caused by heating and their influence on heat stability, whereby such changes tend to increase (↑) or reduce (↓) heat stability or impact is unknown (?).

Heat-induced change	*Accompanying change and its relevance to heat stability*
Denaturation of whey proteins and interactions with κ-casein	At < pH 6.7 complexation of β-lg and α-la onto casein micelle surface (↑) At > pH 6.7 formation of soluble complexes between β-lg and κ-casein (↓)
Dephosphorylation of caseins	Possible decline in pH (↓); dissociation of calcium-sensitive caseins (?)
Conversion of lactose into organic acids	Decline in pH (↓)
Conversion of soluble primary calcium phosphate to insoluble tertiary calcium phosphate: $3CaH_4(PO_4)_2 \rightarrow 3CaHPO_4 + 3H_2PO_4^- + 3H^+$ $\rightarrow Ca_3(PO_4)_2 + 2H_2PO_4^- + 5H^+$	Decline in pH (↓); precipitation of calcium phosphate with indigenous CCP (?) or onto casein micelle surface (?) or out of solution (?)
Intermicellar covalent bond formation: thiol bridges, lysinoalanine, lanthionine	Irreversible aggregation of casein micelles (↓)
Hydrolysis of κ-casein	Loss of intermicellar steric and electrostatic repulsive forces (↓)
Maillard reaction	(?)
Formation of lactulose	(?)

4.1 Interactions between whey proteins and κ-casein

The most critical factor which determines the shape of the HCT/pH profile of type A milk is probably the influence of pH on thermal interactions between whey proteins and κ-casein, as depicted in Figure II-7. Using gel permeation chromatography, Singh and Latham (1993) isolated complexes between α-la, β-lg and κ-casein in heated (140°C) milk; the size of these complexes was estimated to be ~ 10^6 Da. These authors observed that when the initial pH of the milk was in the range ~ 6.3 - 6.7, complexes between whey proteins and κ-casein were mostly on the surface of the casein micelles, whereas at > pH 6.7, there was an increased tendency for the complexes to become soluble; at initial pH 6.67, it was estimated that ~ 85 - 90% of whey protein/κ-casein complexes were on the micelle surface, while at pH 7.2, ~ 50% of the complexes were soluble. At the higher pH values, increased electrostatic repulsive forces were probably responsible for promoting solubilization of the

whey protein/κ-casein complexes. Singh and Latham (1993) found also that the addition of mercaptoethanol to milk inhibited the formation of the complexes, indicating that the thiol groups of α-la, β-lg and κ-casein were involved in complex formation.

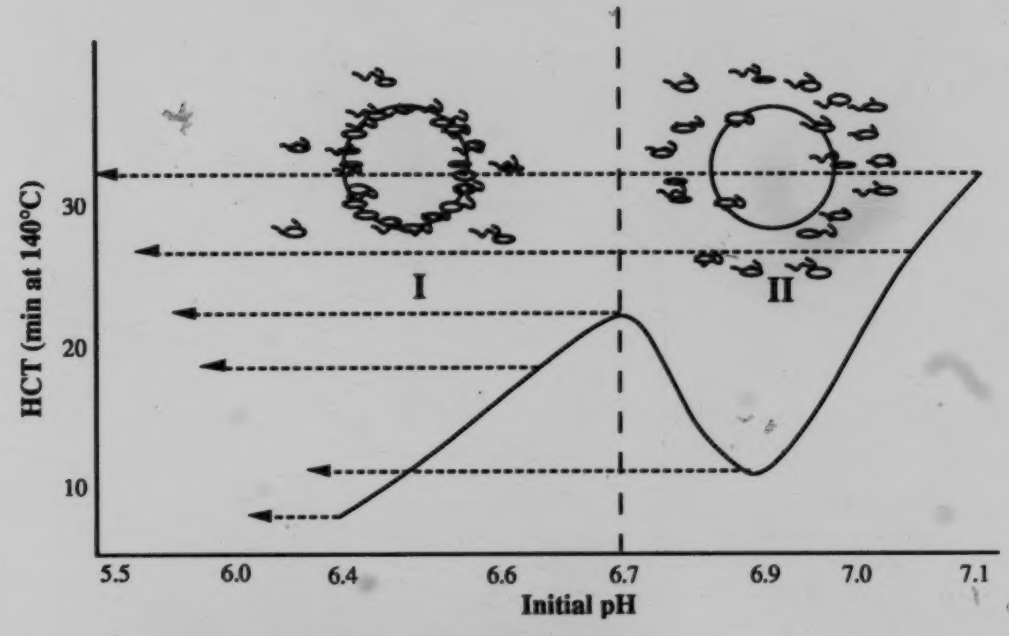

Figure II-7. Schematic illustration of pH-dependent interactions between κ-casein and β-lactoglobulin caused by high temperature heating of milk, and the approximate extent of pH decline in milk heated at different initial pH values to its coagulation point.

The work of Singh and Latham (1993) confirmed the earlier investigations of Kudo (1980) and Singh and Fox (1985a, b, 1986, 1987a, b), where gel electrophoresis showed the absence of β-lg/κ-casein complexes in the ultracentrifugal supernatant of milk heated at initial pH ≤ 6.7, suggesting that whey proteins had co-sedimented with the caseins, whereas a significant concentration of β-lg/κ-casein complex occurred in the ultracentrifugal supernatant of milk heated at initial pH > 6.7, indicating the formation of soluble complexes.

Using electron microscopy, Mohammad and Fox (1987) showed that heating milk, at initial pH 6.6 - 6.7 and at 130 - 140°C for 10 min, caused an increase in the average micelle diameter; this was attributed to the deposition of whey proteins onto the micelle surface, revealed by the presence of numerous appendages protruding from the surface of the micelles. At higher pH values, Mohammad and Fox (1987) found that heating of milk led to a decrease in the average micelle diameter, which appears to be consistent with the loss of κ-casein from the surface of the micelle, due to its complexation with β-lg. Mohammad and Fox (1987) observed also that when milk was heated, at initial pH 6.7, to its coagulation point, the coagulum consisted of short chains of casein micelles, which formed an open network; at initial pH 6.9, a much denser network was apparent, consisting of closely packed, fused casein micelles.

The heat stability of casein micelles dispersed in milk ultrafiltrate, or of milk in which the whey proteins have been removed, shows a continuous increase with pH (Figure II-6); compared to normal type A milk, these systems are less heat stable at ≤ pH 6.7, but more heat stable in the region of the minimum. Therefore, the presence of complexes between whey proteins and κ-casein on the surface of the casein micelle increases heat stability, whereas formation of soluble complexes has the opposite effect. Further evidence for this hypothesis was provided by Tessier and Rose (1964), who showed that enrichment of type A milk with κ-casein eliminated the minimum in the heat stability curve, probably because the presence of the extra κ-casein in the serum competed against κ-casein located on the surface of the casein micelles for the binding of β-lg and hence inhibited the dissociation of micellar κ-casein. Singh and Fox (1985b) postulated that the existence of surface-micellar complexes between whey proteins and κ-casein increased micellar charge, ζ-potential and hydration, improving micelle resistance to heat coagulation. This view was supported by the works Schmidt and Poll (1986) and Anema and Klostermeyer (1996) who found that the ζ-potentials of casein micelles were increased by their thermal complexation with whey proteins. At > pH 6.7, the heat-induced formation of soluble whey protein/κ-casein complexes causes depletion of κ-casein from the micelle surface. Depletion of κ-casein reduces greatly the heat stability of casein micelles, probably due to a combination of reduced electric charge and hydration at the micelle surface and a loss of the steric repulsion due to the loss of the glycomacropeptide portion of κ-casein. This effect is most evident when the initial pH of milk is 6.9;

at > pH 6.9, κ-casein-depleted micelles display greater heat stability due to the increasing intermicellar electrostatic repulsive forces. It was proposed by van Boekel *et al.* (1989) that when the initial pH of milk was > 6.9, during the initial stages of heating soluble complexes between whey proteins and κ-casein formed, but the complexes subsequently associated onto the surface of the casein micelles, contributing also to an improvement in heat stability.

A distinct feature of the heat stability profile of type A milk is the abrupt decline in heat stability when the initial pH is increased above 6.7. This indicates a very sharp increase in the degree of dissociation of β-lg/κ-casein complexes from the casein micelle. If the extent of dissociation of β-lg/κ-casein complexes from the casein micelle was governed merely by the extent of electrostatic repulsive forces, then a more gradual decline in heat stability would be expected at pH > 6.7. An explanation for the extreme sensitivity of the heat stability of type A milk to pH, at pH > 6.7, could be the 'Tanford' transition of β-lg. It was observed by Tanford *et al.* (1959) that in the pH range ~ 6.0 - 8.5, β-lg underwent a reversible conformational change from the 'N' to the 'R' state. Later, Dunnill and Green (1966) proposed that this conformational change may have been related to the deprotonation of a histidyl residue and was accompanied by an increase in the thiol group reactivity of β-lg. These authors showed that the reaction of para-chloromercuribenzoic acid with the sulphydryl groups of β-lg was a second order process; notably, the value of the rate constant for this reaction displayed a 13 fold increase, when the pH was adjusted from 6.75 to 7.05.

4.2 Decline of pH

Severe heating of milk causes a significant and irreversible decline in pH, which is essential for the onset of protein coagulation; if milk is heated at 140°C, periodically withdrawn and the developed acidity neutralized, extremely high (> 3 h at 140°C) stability results (Pyne, 1958; Fox, 1981). Thus, although the initial pH of milk has a profound effect on heat stability, this is not the pH at which coagulation occurs. A pH decline is essential for the heat-coagulation of casein micelles, which suggests that a certain degree of charge that creates electrostatic repulsive forces, probably on the surface of the micelles, must be neutralized by protonation, before coagulation can occur.

The heat-induced acidity in milk may be caused by three changes (Table II-9), as outlined by van Boekel *et al.* (1989) and Singh and Creamer (1992): (1) The degradation of lactose into organic acids, mainly formic acid, causes ~ 50% of the pH decline; (2) The conversion of soluble primary calcium phosphate into secondary and tertiary calcium phosphates with release of H^+ ions, contributes to ~ 30% of the pH decline; (3) Hydrolysis of the ester bonds of casein phosphoseryl residues and the subsequent reaction of the released phosphate with calcium to tertiary calcium phosphate with release H^+ ions, may be responsible for ~ 20% of the pH decline; however, the work of Dalgleish *et al.* (1987) casts doubt on this premise - see section 4.5.

It has not been possible to determine the pH of milk during heating at high temperature; instead, the pH of heated and subsequently cooled milk has been measured. This is not entirely satisfactory, as the pH of milk during heating would probably be lower than that of heated and cooled milk; cooling of milk would be expected to cause some resolubilization of heat-precipitated calcium salts tending to increase the pH (see section 2.3.2). In the case of milk which had been heated at 140°C and cooled to temperatures in the range 60 - 90°C, an approximately linear relationship between the temperature of cooling and the pH of milk occurred (Fox, 1982). By extrapolation of the data, it was estimated that when milk was heated at 140°C, at its natural pH (~ 6.7), to the point of coagulation (~ 20 min) the pH would decline to ~ 4.9. A time dependent pH decline upon heating of milk was demonstrated by van Boekel *et al.* (1989); when milk was heated, at 130 or 140°C, over the initial pH range ~ 6.3 - 7.1, a rapid decrease in pH occurred during the initial 2 min of heating, after which a more gradual decline in pH took place and the initial pH did not seem to have a strong effect on the rate of pH decline. These authors proposed that the initial decline in pH was caused by precipitation of calcium phosphate, whereas the more gradual decrease was due to the formation of organic acids from lactose.

Although a pH decline is essential for the heat-coagulation of milk to occur, it is clear that coagulation is not merely a type of acid-coagulation because, as outlined by van Boekel *et al.* (1989) and Singh and Creamer (1992): (1) Increasing the pH of milk which has been heat-coagulated does not lead to dissolution of the protein clots; (2) The pH decline has a Q_{10} of ~ 2, whereas heat coagulation has a Q_{10} of ~ 3; (3) Depending on its initial pH, milk coagulates at different pH values. These observations point to the

involvement of other types of forces in heat coagulation, which probably involve salts and the formation of covalent bonds, as discussed in the next sections.

4.3 Changes in salt equilibria

It is generally accepted that heating of milk leads to a shift in salt equilibria, due to the conversion of soluble monocalcium phosphate to insoluble di- and tricalcium phosphates; this is responsible for part of the heat-induced pH decline, which in turn contributes to reduced heat stability, as described above. However, as described in section 2.3.2, the fate of heat-precipitated calcium and phosphate is uncertain; it may precipitate with indigenous CCP, to increase the size of CCP nanoclusters; it may associate onto the surfaces of the casein micelles; or it could merely fall out of solution.

If calcium and phosphate are deposited onto the casein micelle surfaces during heating of milk, then a reduction in intermicellar electrostatic repulsive forces and hence heat stability might be expected. Such a destabilizing effect might be especially pronounced in the vicinity of the minimum, where the electrostatic and steric stabilizing effect of κ-casein is lost. Morrissey (1969) demonstrated that a modest reduction (from 13 to 11 mM) of the soluble calcium and soluble magnesium content of milk caused a large increase in heat stability in the zone of the minimum (initial pH ~ 6.9), alluding that κ-casein-depleted micelles are extremely sensitive to changes in salt composition. Fox and Hearn (1978) dialyzed milk against distilled water to reduce its content of soluble calcium and phosphorus and found a similar increase in heat stability near initial pH 6.9.

It appears that heating does not change the solubility of the monovalent ions sodium, chloride and potassium and most studies also indicate that the state of citrate is not greatly altered by heating (Singh and Creamer, 1992).

4.4 Polymerization reactions

The inability to dissolve heat-aggregated casein by adjustment of pH, indicates that intermicellar aggregation cannot be governed by electrostatic forces alone, whether these forces be modulated by changes in pH or salt equilibria, and other types of forces, especially covalent, must also be involved. Figure II-8 indicates the various types of covalent bonds which could be involved in the heat-induced polymerization of casein.

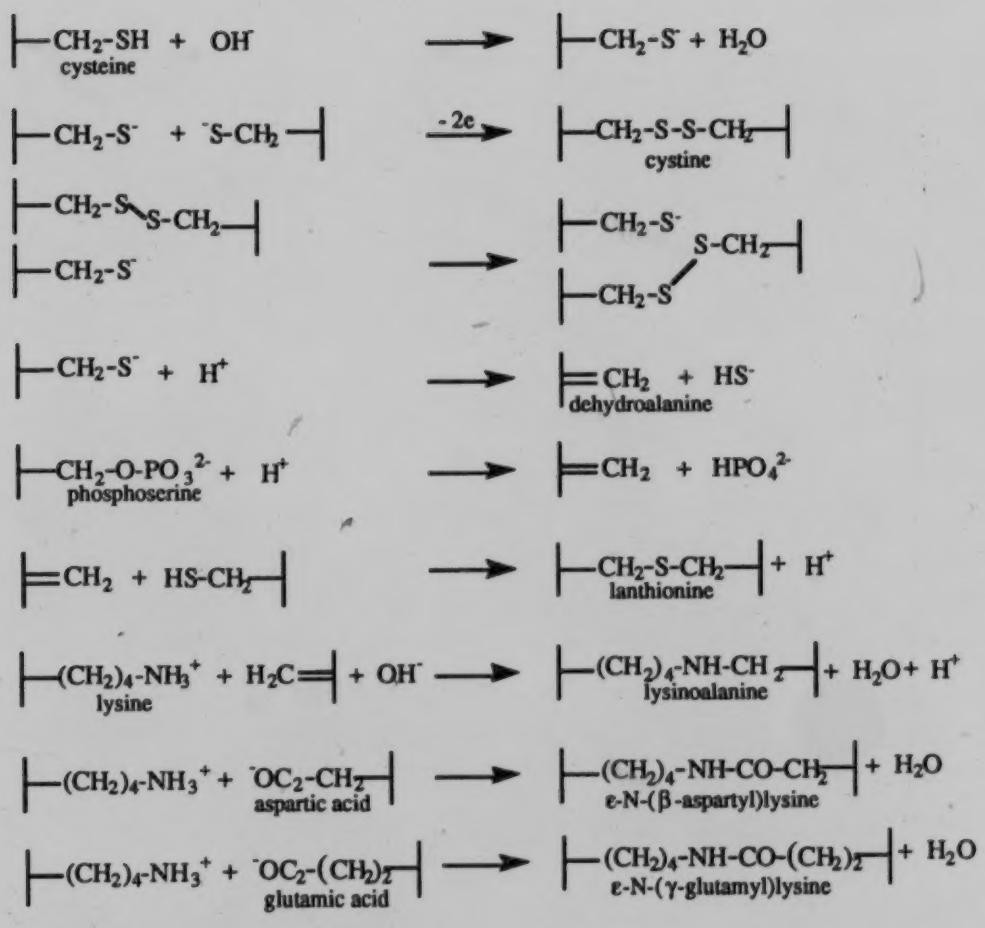

Figure II-8. Thermal reactions leading to formation of covalent bonds between proteins. Vertical bars indicate attachment of groups to the amino acid backbone of the protein. Adapted from Walstra and Jenness (1984).

It was attempted by van Boekel *et al.* (1989) to determine the nature of the forces between heat-aggregated casein molecules; milk samples, adjusted to initial pH values in the range 6.2 - 7.1, were heated to their coagulation points and each coagula mixed with a solution of mercaptoethanol (which disrupts sulphydryl bridges) or oxalate (which sequesters calcium). At initial pH 6.2, oxalate was capable of dissolving the coagulum, indicating that electrostatic forces, mediated by calcium, were needed for the formation of coagula. At all pH values > 6.2, none of the coagula could be dissolved by mercaptoethanol or oxalate. This observation did not rule out a role for electrostatic forces,

as well as the formation of covalent bonds between cysteinyl residues, for the polymerization of casein to occur, but also indicated that the formation of a novel-type of covalent bond was involved. The nature of this novel-type of covalent bond was not established by van Boekel *et al.* (1989), but the formation of lysinoalanine or lanthionine were speculative possibilities.

Shalabi and Fox (1982a) reported that various carbonyl compounds, especially in the presence of urea, dramatically increased the heat stability of milk. These authors suggested that the ε-amino groups of lysyl residues contributed to the heat sensitivity of milk by participating in cross-linking reactions between the caseins; these reactions were prevented when an ε-amino group reacted with a carbonyl compound, the reaction being catalyzed by urea. Later, Shalabi and Fox (1982b) suggested that arginyl residues might also play a role in the heat coagulation of milk; carbonyl compounds, in the presence of urea, may have reacted with these residues, in a similar manner to lysyl residues, to increase the heat stability.

4.5 Other physico-chemical and structural changes of casein micelles

A number of other changes occur to the structure of the casein micelles upon high temperature heating of milk, which include dephosphorylation, dissociation of calcium-sensitive caseins and hydrolysis of κ-casein; the impacts of these changes on heat stability are less well established.

Dalgleish *et al.* (1987) showed that about two thirds of the phosphates attached to the seryl residues of the caseins were hydrolyzed after 30 min at 130°C. One effect of this dephosphorylation would be its possible contribution to the pH decline upon heating, as already described in section 4.2. Also, as proposed by Dalgleish *et al.* (1987), it is possible that dephosphorylation would lead to dissociation of the caseins from the micelle, considering that their attachment to CCP nanoclusters would be lost. Dissociation of the calcium-sensitive caseins may well be related to their dephosphorylation, but it is less likely to be the case for κ-casein; Singh and Creamer (1992) considered that dissociation of κ-casein from the micelle (at > pH 6.7) was not related to dephosphorylation, because the phosphoseryl groups of κ-casein are probably not attached to calcium-phosphate nanoclusters and the rate of κ-casein dissociation is more rapid than the rate of dephosphorylation. Considering that at > 110°C, hydrophobic forces are so weakened as

to become practically non-existent (Singh and Creamer, 1992), it is likely that dissociation of all of the four casein types is promoted by a loss of hydrophobic forces.

Dalgleish *et al.* (1987) showed that dephosphorylation of the caseins did not lead to an increase in the concentration of non-sedimentable phosphate, indicating that the effect of dephosphorylation would be to convert organic phosphate to inorganic phosphate and the structure of indigenous CCP would be maintained; if this is true, then dephosphorylation would not contribute to the heat-actuated pH decline in milk.

When milk was heated at its unaltered pH at 85°C for 10 min, and stored at 30 °C for 22 h, the concentration of serum casein increased; ~ 23, 4, 4 and 3% of total κ-, β-, α-$_{s1}$- and α-$_{s2}$-casein, respectively, became dissociated (Law, 1996). Heating of milk at its unaltered pH probably causes formation of complexes between κ-casein and β-lg on the surface of the casein micelle, improving heat stability, but the work of Law (1996) suggests that dissociation of κ-casein may occur at the same time. Little concrete information is available as to how dissociation of the calcium-sensitive caseins from the micelle may affect the heat stability of milk.

Hindle and Wheelock (1970) reported that heating milk, at its unaltered pH (~ 6.7), at 50 - 100°C, for up to 8 h, led to an increase in its non-protein-nitrogen content, especially as the temperature and duration of heating increased; unheated milk contained ~ 2 mg 100 mL^{-1} of non-protein nitrogen, compared to ~ 16 mg 100 mL^{-1} in milk heated at 100°C for 8 h. The peptides produced were similar to the glycomacropeptide of κ-casein produced by the action of chymosin, though they had a lower content of carbohydrate, probably because heating also caused cleavage of the carbohydrate groups from the liberated peptides. Considering that the loss of gylcomacropeptide from casein micelles, by the action of chymosin, leads to micelle coagulation, it is likely that the heat-induced proteolysis of κ-casein plays an important, and perhaps underestimated, role in the heat-coagulation of casein micelles.

4.6 Maillard reaction

When milk is heated at a high temperature, the Maillard reaction (MR) takes place to a significant extent, alongside the various other thermal reactions. In milk, the MR is initiated primarily by the reaction of the electrophilic ε-amino group of lysine with the nucleophilic carbonyl group of lactose, which is the initial step leading to the formation of a

complex 'labyrinth' of compounds. The whey proteins and α-$_{s2}$-casein have the highest content of lysine and therefore would be expected to be the most active participants in the Maillard reaction. The detailed chemistry of the MR has been reviewed (O' Brien, 1995) but very little is known of its importance, if any, in the heat coagulation of milk. The rate of the MR increases as the pH of milk is increased, but it is not known whether this affects heat stability. It might be expected that the MR would tend to increase heat stability, because the reaction of lysine with lactose would make lysine less available for participation in covalent reactions which could be involved in casein polymerization, but evidence for this hypothesis is lacking (Fox, 1982).

4.7 Heat stability of type B milk

Samples of milk from individual cows usually display type A behaviour, but a significant number are of type B, where no minimum exists and heat stability increases gradually with increasing pH (Figure II-6). Sweetsur and White (1974) reported that the addition of 0.5 - 2.0 mg mL^{-1} of β-lg to type B milk caused its heat stability/pH curve to more closely resemble that of type A milk, while enrichment of type A milk with β-lg accentuated the minimum. This indicates that the ratio of β-lg to κ-casein in milk may be the critical factor governing its heat stability/pH profile; in type A milk, complexation of β-lg with κ-casein promotes dissociation of κ-casein in the region of the minimum, but in type B milk the concentration of β-lg is perhaps too low for this destabilizing reaction to occur to a significant extent. This hypothesis is supported by the work of Tessier and Rose (1964) who reported that enrichment of type A milk with κ-casein eliminated the minimum of the heat stability curve, probably because the presence of extra soluble κ-casein impeded dissociation of micellar κ-casein by competing for complexation with β-lg. At < pH 6.7, type B milk is less heat stable than type A milk, possibly because of the absence of a micellar surface complex between β-lg and κ-casein.

Sweetsur and White (1974) observed that when the heating temperature was lowered from 140 to 130°C, the minimum of type A milk was eliminated so that its heat stability/pH profile resembled that of type B milk. The reasons for this were unclear, but the result indicates that a relatively high activation energy is needed for the formation of soluble aggregates between whey proteins and κ-casein.

4.8 Heat stability of concentrated milk

A large amount of effort has been devoted to elucidate the mechanisms responsible for the heat stability of unconcentrated milk. Much fewer studies have been carried out on the heat stability of concentrated milk, even though this is of significant practical importance; the heat stability of concentrated milk is frequently too low to withstand industrial heat treatments, such as in-can sterilization (110°C/5 - 10 min). Probably, the 'maze' of thermal reactions which occur during heating of unconcentrated milk, also take place during heating of concentrated milk and these are accentuated by the partial removal of water which increases the concentration of all reactants. Bulk milk is almost invariably of type A, implying that all studies on the heat stability of concentrated milk have been carried out with concentrate prepared from type A milk, though this is rarely stated in the literature. In Figure II-9, a typical heat HCT/pH profile for a type A milk concentrated to ~ 20% total solids is shown.

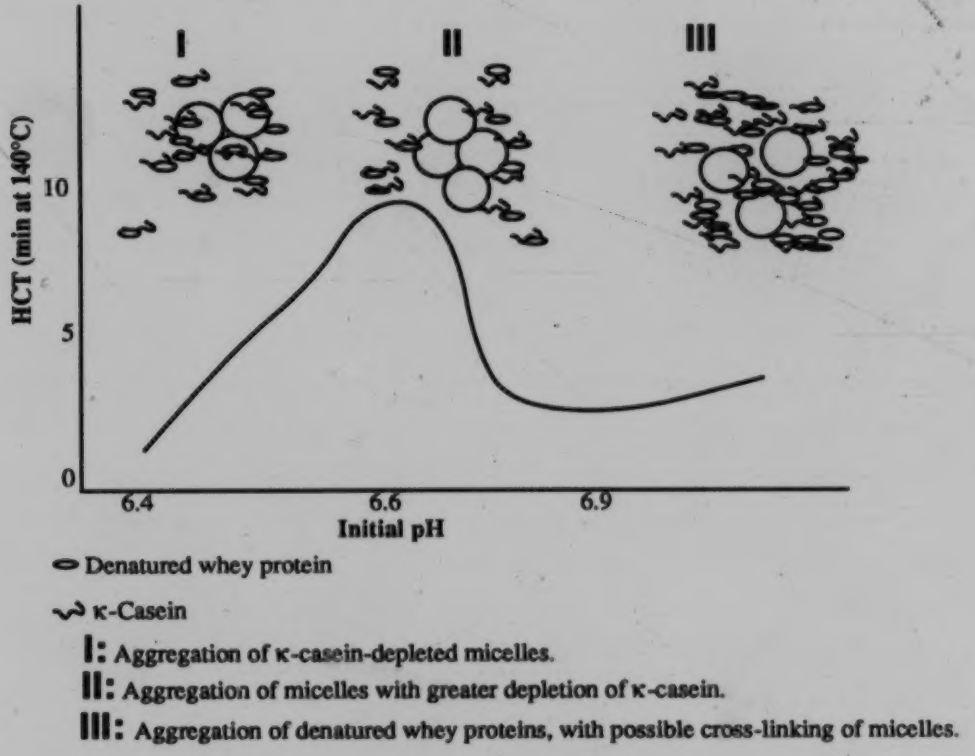

Figure II-9. A typical heat stability/pH profile for a type A milk concentrated to 20% total solids and the possible types of protein aggregates formed upon heating at different initial pH values. Adapted from Singh *et al.* (1995).

The general effect of concentration is to reduce greatly the heat stability, though a small maximum occurs at ~ pH 6.6. Concentration of milk causes a closer packing of casein micelles, a significant pH decline and an increased activity of calcium and magnesium ions, all of which would be expected to reduce heat stability. Singh and Creamer (1992) and Singh *et al.* (1995) proposed that, depending on the initial pH of concentrated milk, three types of coagulation processes could happen (Figure II-9).

At initial pH < 6.4, coagulation involves the aggregation of κ-casein-depleted micelles, promoted by the formation of salt bridges involving calcium or calcium phosphate. Loss of κ-casein is due to the formation of soluble κ-casein/whey protein complexes, similar to that which occurs in the zone of the minimum for unconcentrated milk, except it occurs at a lower initial pH value. At initial pH 6.5 - 6.9, a greater loss of κ-casein from the micelle surface occurs, but heat stability increases, due to increased intermicellar charge repulsion. At initial pH > 6.7, the casein micelles are thought to become highly dissociated and coagulation could involve the formation of a gel network between whey proteins and dissociated κ- and calcium-sensitive caseins.

In industrial situations, it is a common practice to preheat milk (1 - 3 min at 110 - 120°C) prior to its concentration, followed by the addition of various types of salts (see section 2.3.4) to the concentrated milk, to improve its heat stability during ultra-high temperature heating or in-can sterilization. The effects of pre-heating and added salts on the heat stability of concentrated milk are not well understood and in practice optimization of heat stability is achieved by trial and error. It is possible that pre-heating causes association of whey proteins onto the casein micelle surface which inhibits the formation of whey protein/κ-casein complexes during the subsequent more intense heat treatment of the concentrated milk. Augustin and Clarke (1990) showed that the effects of the addition of phosphate salts to concentrated milk were to reduce calcium ion activity, increase the concentration of colloidal phosphate and possibly to buffer against a pH decline during heating, but it was difficult to correlate these changes to the observed heat stabilizing effect.

Sweetsur and Muir (1980) compared the heat stabilities of normal skim milk (~ 9.5% total solids) and skim milk concentrated by either evaporation or UF up to 18.4% total solids. In the initial pH range 6.4 - 7.2, concentrated or ultrafiltered milk samples were much less heat stable than normal milk, but the ultrafiltered milk was still about twice as heat stable as the concentrated milk; for example, at pH 6.8 the HCT's of normal,

ultrafiltered or evaporated milk were ~ 40, 25 or 10 min, respectively. The protein contents of the concentrated or ultrafiltered milk products were ~ 7 or 13%, respectively, which indicated that a higher ratio of protein to non-protein solids increased the heat stability. A reduced ratio of soluble salts to protein might lead to increased heat stability, if the heat-induced precipitation of calcium phosphate salts was reduced and thus the extent of the consequent pH decline. It was reported also by Sweetsur and Muir (1980) that preheating (90°C/10 min) of concentrated milk almost doubled its heat stability, but this treatment had little effect on the heat stability of ultrafiltered milk, but no explanation could be offered for this observation.

5 Protein standardization of milk and dairy products*

In the dairy industry, for many decades, it has been a common practice to standardize the fat content of milk. This has developed because until recently milk fat has been considered to be the most valuable milk constituent and its concentration can be altered easily by mechanical means - the centrifugal separator. In many countries, the consumer has a choice of milk with no fat, reduced fat (1 - 2%) or natural fat content (~ 3.5 - 4.0%). The surplus fat resulting from the 'down' standardization process is used to manufacture butter, cream and other high-fat dairy products. It is well known that the fat content of bovine milk varies widely due to breed, lactation stage, nutritional status of the cow, climatic conditions, farming practices and other factors. There is no legal definition of raw milk with respect to the minimum fat content and thus the fat standardization of fluid milk always means 'down' standardization.

Standardization of other milk components is much less common. The manufacture of condensed milk or milk powder might be considered as 'standardization' of total solids, by removal of water, without alteration of the dry matter composition. The addition of β-galactosidase to milk to hydrolyze lactose into glucose and galactose, to enable consumption by lactose intolerants, might be termed 'down standardization' of lactose. Recently, a fluid milk product was introduced by the Toni Lait company to the Swiss market under the trade name 'Sport Milch'; this may be viewed as fluid milk standardized 'upward' with various vitamins and minerals (Henck, M., personal communication).

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* A version of this section was published: Rattray, W. & Jelen, P. (1996). *Trends Food Sci. Technol.* 7, 227-34

Similar milk products exist in other countries, for example 'Physical' in Australia; most of these products have been 'up'-standardized especially with respect to calcium.

Although to a lesser magnitude than in the case of fat, the protein content of fluid milk also exhibits considerable natural variability, as indicated (Table II-10) by a recent international survey (Higgins *et al.*, 1995). Even relatively small natural variations in protein content can be significantly magnified in dairy products manufactured by removal of water such as skim milk powder. There is now a strong incentive to obviate this variability, by protein standardization (Marshall, 1995), which has become technologically feasible, especially by using UF; technology for continuous standardization of fat and protein in milk, by use of centrifugal separation in combination with UF has been demonstrated by Rønkilde Poulsen (1978) and Friis (1985, 1986). Standardization of condensed milk and milk powder, by the use of UF or addition of isolated lactose, has been approved in principle at the 1995 annual session of the International Dairy Federation (IDF, 1996) and is now the subject of international consultations.

Table II-10. Natural variations in the protein content of milk and milk powder in some countries[1].

Country	*Milk* (*g protein 100 g^{-1} milk*)	*Milk powder* (*g protein 100 g^{-1} solids-not-fat*)
Austria	3.21 - 3.46	34.3 - 37.0
Belgium	3.18 - 3.53	35.7 - 38.2
Canada	2.75 - 4.09	No data
France	3.09 - 3.38	35.0 - 38.5
Finland	3.11 - 3.40	34.0 - 37.0
Germany	3.26 - 3.48	No data
Ireland	2.85 - 3.60	33.5 - 40.8
New Zealand	3.16 - 4.22	33.5 - 41.0
Norway	3.16 - 3.33	36.4 - 37.5
Spain	2.80 - 3.25	No data
United Kingdom	2.96 - 3.54	33.8 - 38.9

[1]: Adapted from Higgins *et al.* (1995), where more comprehensive data can be found.

It must be emphasized that the term protein standardization, as interpreted by the IDF, implies relatively small changes in protein content, within the limits of natural variability, achieved by the addition or removal of natural milk constituents, without changing of the ratio of whey protein to casein. In this section, the term 'protein adjustment' is also used, indicating more drastic alteration of the milk protein component, which may or may not be accompanied by a change in the ratio of casein to whey protein.

Protein standardization would offer many advantages to producers, processors and consumers of milk, but its essential purpose is to minimize the natural variability of milk protein content. Protein adjustment is most beneficial to milk processors and consumers of dairy products and may be carried out to improve the nutritional and/or physico-chemical properties of milk and other dairy products.

No specific regulations allowing for protein standardization on a global basis are currently available. As a result, consumers purchase fluid milk and other dairy products, whose nutritional value may vary with regional, seasonal and other factors, similar to those which influence the fat content. Protein standardization would improve the nutritional uniformity of milk and other dairy products. Unlike fat standardization, a minimum protein content of cow milk is defined in some jurisdictions; in the European Union, the minimum standard is 2.8% (w/v) total protein (N x 6.38). Thus, the notion of protein standardization, as currently envisaged, would entail not only 'downward' standardization but also 'upward' standardization of fluid milk to meet the required minimum protein content, depending on whether the natural protein content of the milk was above or below the specified minimum. Protein standardization 'upward' would allow some countries to meet specifications for condensed milk and milk powders, required by international markets, which could not otherwise be met due to the naturally low protein content of their milk. The proposed methods of protein 'down' standardization, by addition of milk UF permeate or lactose, allow for convenient utilization of by-products of the dairy industry, thus leading to a potential decrease in the price of 'down'-standardized milk for the consumer.

The economic implications of protein standardization have not been fully assessed, but a recent calculation (Hyborg, 1995) indicated that if standardization of fluid milk to 3% protein and milk powder to 34% protein in solids-not-fat were to be practiced in the European Union, up to 128 x 10^6 kg of extra protein, or ~ 4% of total milk protein produced, would be available for processing into other dairy products. Milk producers might be able to obtain a more accurate price; milk with a naturally high protein content should command a higher selling price. Similarly, processors of milk would recover their costs of raw milk purchases based on the protein content of final products, as is presently the case regarding milk fat.

Protein standardization could have environmental benefits. For example, addition of UF permeate to milk for 'down' standardization would eliminate pollution arising from disposal of untreated UF permeate, which has a high biological oxygen demand, or would reduce consumption of energy required for its treatment or processing.

5.1 Methods available to alter the protein component of milk

Alteration of the milk protein component can be carried out at the production stage, by interfering with the biochemical processes which enable the cow to produce milk, or by post production, technological means. The principal methods available for protein standardization or adjustment in milk and their basic effects on the protein fraction are shown in Table II-11. Methods used at the production stage have focused on manipulating the genes involved in milk protein biosynthesis or modification of the nutrient intake of the cow. These techniques do not usually offer immediate flexibility to the processor and fall outside the terms 'protein-standardization' or 'protein adjustment', as defined above, because the 'natural' protein content of the milk is maintained.

5.1.1 Genetic manipulation

In principle, genetic manipulation is the most powerful method to modify the protein component of milk, but in practice it is difficult to implement and much more has been proposed than has been actually achieved. The genes encoding for the 6 major bovine milk proteins, namely, α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin have been identified and considerable progress has been made to elucidate their mode of expression (Martin and Grosclaude, 1993). Genetic engineering could involve increasing total milk protein concentration and/or altering its composition. This could be achieved by changing the number of genes involved in the synthesis of specific proteins or by interfering with the mode of gene expression. Specific proposals include elimination of β-lactoglobulin from bovine milk, to annul its allergenicity, or increasing the κ-casein content to increase the heat stability of milk (Martin and Grosclaude, 1993). Site-directed mutagenesis offers the possibility to change the amino acid sequence of milk proteins and hence their tertiary structures and physico-chemical and functional properties. Naturally occurring genetic polymorphs of milk proteins, especially the caseins and β-lactoglobulin, differ in some of their physico-chemical properties and thus their varying

concentration in milk can affect technological properties, including heat stability and rennetability (Jakob and Puhan, 1992); this has stimulated interest in altering the proportions of genetic polymorphs in milk. The use of transgenic animals to produce milk with the same composition as human milk is the subject of intensive and ambitious studies (Martin and Grosclaude, 1993).

Table II-11. Some methods available to alter the protein fraction of fluid milk and their effects on protein concentration and composition.

Method	*Total protein*	*Ratio of whey protein to casein protein*
Biosynthetic		
Genetic manipulation	Increased	Increased or reduced
Nutritional manipulation	Increased	Usually unchanged
Technological		
UF of milk[1,2]	Increased	Unchanged
Addition of milk UF retentate[1,2]	Increased	Unchanged
Addition of milk UF permeate[1]	Reduced	Unchanged
Addition of isolated lactose[1]	Reduced	Unchanged
Addition of water	Reduced	Unchanged
Addition of UF permeate, derived from sweet whey or acid whey	Reduced	Unchanged
Addition of dried or liquid whey protein products; whey protein concentrate, whey protein isolate, whey protein fraction	Increased or unchanged	Increased

1: These methods are currently most favoured by the IDF for protein standardization.
2: The UF of milk or the addition of UF retentate to milk are mutually equivalent.

5.1.2 Nutritional manipulation

Progress in nutritional science has enabled producers to alter feeding practices for increased milk protein production. The amino acids used to synthesize bovine milk protein originate from undegraded dietary protein reaching the duodenum of the lactating cow. Increasing the protein content of milk can be achieved by increasing the supply and/or altering the

profile of amino acids in the duodenum (Murphy and O' Mara, 1993). To increase protein synthesis, the energy, protein and amino acid content of feed needs to be increased, while lipid content is reduced. The composition of forage affects protein synthesis in a similar manner. In general, nutritional manipulation is easier to carry out than genetic engineering, but can only be used to increase total protein without altering the composition of the protein fraction or the properties of specific proteins.

5.1.3 Technological methods

Technological methods for altering the protein composition of milk are diverse and offer more immediate flexibility to the milk processor. The UF of milk is a complex physico-chemical process, but in essence involves the pressurized flow of milk through a membrane, which is porous to low MW compounds, but retains most of milk protein and fat. The retained portion of milk, termed the retentate or ultrafiltered milk, has an increased ratio of total milk protein (and fat if present) to low MW milk solutes, while the ultrafiltrate or permeate is an aqueous solution of lactose, salts, vitamins and other low MW compounds. Information on the principle of UF and its use in the dairy industry is extensive (Renner and Abd El Salam, 1991; Rosenberg, 1995). Figure II-10 shows how UF could be used to produce protein 'up'- or 'down'-standardized skim milk.

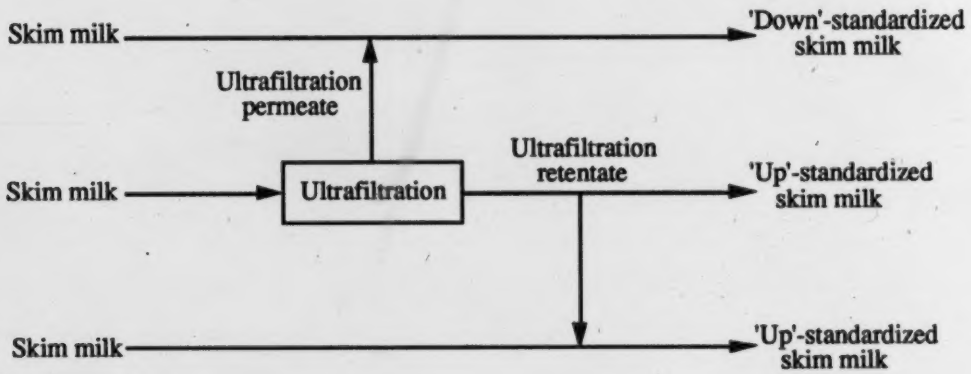

Figure II-10. Schematic illustration of the use of UF for the production of protein-standardized skim milk. More complicated flow diagrams showing the simultaneous standardization of fat and protein in milk were provided by Friis (1985, 1986).

Milk UF retentate can be used either directly or after blending with other milk to achieve 'up' standardization. The addition of milk UF permeate to bulk milk will result in 'down'-standardized milk. The use of UF for protein standardization involves merely the physical separation of milk into protein-enriched and protein-free streams, analogous to the separation of whole milk into cream and skim milk (Lankveld, 1995), and does not alter the natural ratio of whey protein to casein in milk. Other UF permeate types which might be suitable for protein standardization 'downward', include permeate made by UF of sweet whey or of acid whey produced either by direct acidification or by fermentation of skim milk, though at present the IDF recommends the use of milk UF permeate only for 'down' standardization.

The use of isolated lactose is another method of protein 'down' standardization which was approved by the IDF for standardization of condensed milk and milk powder, without the need for labeling as an extra ingredient (IDF, 1996); this is a little controversial. Although the amount of added lactose to be used for protein standardization is likely to be small, it could be argued that this is similar, in principle, to the addition of water, because the addition of a single component dilutes the concentration of all other milk nutrients. Unlike protein standardization by the use of UF procedures, addition of lactose involves an ingredient which, although natural and unique to milk, normally does not originate from the milk being standardized, but from a different source (usually cheese whey). Thus, the use of lactose for any protein standardization, including the already approved condensed and dried milk, should require labeling. The technique of adding lactose to fluid milk would necessitate dissolution in a suitable aqueous system, either the milk being standardized or an UF permeate. Using water for lactose dissolution would add yet another component to milk; in the case of fluid milk this would in fact violate the conventional view of considering added water as adulteration.

The addition of water to milk for 'down' standardization of protein is not entirely inconsistent with other methods of 'down' standardization; water is a natural constituent of milk and there are a number of sources of milk-derived water, such as permeate from reverse osmosis or condensate from evaporation (Burgess, 1995). Acceptability of this procedure is doubtful, because addition of water reduces the concentration of all other milk nutrients and the sale of milk with extraneous water has always been prohibited.

The addition of caseinate to milk for 'up' standardization has been cited (Burgess, 1995), but the idea may be misguided, considering that this would alter the whey protein/casein ratio and that caseinate is not a natural milk constituent.

Protein adjustment 'upward' can be achieved by the direct addition of dried or liquid milk protein preparations to milk; some of the products available are milk protein concentrate, whey protein concentrate, whey protein isolate or whey protein fraction. In general, in dried form, milk protein concentrate contains ~ 50 - 85% milk protein, whey protein concentrate ~ 35 - 70% whey protein, whey protein isolate > 90% whey protein and a whey protein fraction > 90% protein, the latter product is usually enriched with α-lactalbumin or β-lactoglobulin; the manufacture of these products has been recently reviewed (Jost, 1993; Rosenberg, 1995). The addition of these products to milk is, in effect, an extended use of UF for protein modification, as UF is an essential step used in the manufacture of these products. However, addition of products, in which the whey protein to casein ratio has been altered, would contravene one of the main principles of currently agreed upon protein standardization methods.

While most methods of protein standardization or adjustment are likely to involve the blending of liquids with liquids or liquids with powders, there also exists the possibility of protein standardization of milk powder by blending with other powders and the processing technology has been described (Burgess, 1995). For protein standardization 'upward', this could involve the mixing of regular milk powder with protein rich powders that would not result in a changed ratio of whey protein to casein, whereas for 'down' standardization the blending of milk powder with dried UF permeate or lactose could be considered. Blending of powders is a relatively low cost and flexible technique, but difficulties may arise in achieving an end powder with a homogenous consistency and hence functionality. This would be especially true for blending two powders with substantially different properties, such as dried lactose and dried skim milk.

5.2 Protein-standardized or protein-adjusted dairy products

Protein-standardized fluid milk for direct consumption should closely resemble normal milk, especially in terms of nutritional and sensory properties. On the other hand, protein adjustment may be employed as a pre-treatment of milk to deliberately and favourably alter its functional and/or nutritional properties before conversion into final dairy products.

5.2.1 Fluid milk

At present, standardization of the protein content of pasteurized milk for direct consumption is an important international issue. Standardization of milk protein by the use of UF procedures would appear to be most feasible, as the equipment is available for continuous standardization, adverse changes in heat stability, sensory and nutritional quality are minimal, and there is an analogy between fat standardization and protein standardization by physical fractionation of the original fluid milk and remixing the streams in different proportions. Ongoing discussions indicate that 3% (w/v) minimum protein as a general standard could be acceptable (IDF, 1996), as a compromise between the current legal minimum (2.8%, w/v) in the European Union and the desire of the dairy community to maintain the image of milk as a valuable source of protein. The feasibility of producing protein-standardized, ultra-high temperature heated milk also merits investigation, considering that such milk has a greater consumption than pasteurized milk in many European countries.

Protein adjustment of fluid milk by enrichment with whey proteins could be achieved conveniently by the addition of dried or liquid whey protein concentrate to milk, to increase the nutritional quality of milk. Recent reports of the physiological functionality of whey proteins, including anticarcinogenic and immunomodulatory effects (McIntosh *et al.*, 1995; Wong and Watson, 1995), may provide the incentive to make more drastic alterations of the protein content of milk and other dairy products. At present, there is no indication that any fluid milk products are being sold on the basis of high nutritional value due to an increased whey protein content. However, there has been a recent upsurge in the marketing of various whey protein preparations, as evident by advertising in several popular fitness magazines.

5.2.2 Evaporated milk and milk powder

At the 1995 Annual Sessions of the IDF, protein standardization was approved, in principle, for the manufacture of evaporated milk and milk powder, with the minimum protein concentration in milk powder being set at 34% of the solids-not-fat content (IDF, 1996). In some countries, at certain times of the year, milk has a naturally low protein content and cannot be used to manufacture concentrated milk products such as evaporated milk or milk powder with protein content required by international markets. Thus, it is

proposed that legislation be introduced to allow protein 'up' standardization of milk, by UF procedures, prior to its conversion into evaporated milk or milk powder. The 'down' standardization of milk with a naturally high protein content, by addition of lactose or milk UF permeate, has been also agreed upon. The protein standardization of milk destined for processing into evaporated milk or milk powder could be desirable also for technological reasons, such as control of product viscosity during processing (Marshall, 1995).

5.2.3 Cultured dairy products

It is a current industrial practice to increase the protein content of milk, before its conversion into yoghurt, to obtain a product with improved texture and stability. Usually this is achieved by evaporation, addition of skim milk powder or by UF. It has been claimed that the physico-chemical properties of yoghurt from ultrafiltered milk are superior to those of yoghurt manufactured from evaporated milk or milk containing skim milk powder (Puhan, 1992; Becker and Puhan, 1989; Mistry and Hassan, 1992). When the solids-not-fat content of milk was increased to 9.0, 9.6 or 10.3%, by evaporation, addition of skim milk powder or UF, the yoghurt made from ultrafiltered milk, at all of the three solids-not-fat levels, had the greatest firmness, best nutritional quality and was least prone to syneresis and post acidification, due to a higher protein, calcium and phosphorus content at the same level of total solids (Becker and Puhan, 1989). Ymer is a Danish cultured dairy product, produced conventionally by mixing cream with fermented skim milk. In a modified process, the fermentation of a mixture of ultrafiltered skim milk (~ 6.5% protein) and cream, increased the yield of ymer up to 18% and reduced syneresis, while maintaining the same sensory quality (Olesen Delaney, 1977). The disadvantages of using ultrafiltered milk for the manufacture of cultured dairy products are the increased amounts of milk needed to obtain the same yield of product and possible problems with the utilization of UF permeate. In addition, if the extent of UF is too high, it will result in the development of too firm a texture and a reduced sensory quality.

Another approach to protein adjustment, for the manufacture of cultured dairy products, involves the addition of a protein preparation to milk, including whey protein concentrate, caseinate or milk protein concentrate. A milk protein concentrate, made by UF, diafiltration and spray drying of skim milk, was added to skim milk to increase to 5.6% the total protein content. Fermentation of this milk produced a fat-free yoghurt,

which exhibited no whey separation, avoiding the usual need to add stabilizers (Mistry and Hassan, 1992). An even greater improvement in stability, compared to the use of whey protein concentrate, was claimed by Tomita *et al.* (1991) when milk was fortified with dried whey protein isolate (> 90% protein); separation of whey from set or stirred yoghurt, after 14 days at 5°C, was prevented completely by the addition of whey protein isolate at > 1.5% of total protein, whether the whey protein isolate was added before of after fermentation. Buchheim *et al.* (1986) and Jelen *et al.* (1987) reported that increasing the whey protein content of milk by the addition of whey protein concentrates, derived from sweet or acid whey, allowed production of a sour milk product with substantially reduced viscosity and thus increased drinkability.

5.2.4 Cheese

The manufacture of cheese from milk processed by UF is now well established and two basic strategies are used:

- A modest degree of UF to increase the protein content of milk to the range ~ 4 - 5%.
- More extensive UF of milk to increase protein content to the range ~ 12 - 18%.

The first procedure allows cheese to be manufactured from milk with a more consistent protein content, which would otherwise fluctuate due to seasonal effects and other factors. This improves the consistency and predictability of cheese yield, nutritional value and sensory quality from batch to batch. Optimization of processing conditions during cheese manufacture is easier when the composition of cheese milk is less variable. The increased protein content of the milk reduces the volume of milk needed to make a certain quantity of cheese, thus allowing for more efficient utilization of cheese making equipment. Cheese has been manufactured successfully in this manner in Switzerland for over 20 years (Puhan, 1992).

The essential purpose of the second approach is to increase the yield of cheese. This is caused primarily by a greater retention of whey proteins in the cheese, which would otherwise be lost in the whey; the increased concentration of casein micelles causes greater entrapment of whey proteins and fat globules upon enzymatically-induced gelation. Theoretically, cheese yield could be increased up to ~ 17%, if all the whey was retained (Lelievre and Lawerence, 1988). An increased retention of fat may also contribute to the increased yield, though this is significant only if the cheese is made from homogenized

milk, as is the case for Danish Blue. The disadvantages of making cheese from ultrafiltered milk, as discussed by Lelievre and Lawerence (1988), include: (1) The increased viscosity of the milk hinders homogenous mixing of starter and rennet, which can lead to uneven acid development and entrapment of air bubbles; (2) The extra buffering capacity of the milk proteins makes it more difficult to achieve the desired pH decline; (3) The casein gel tends to be less homogeneous and may lead to uneven cheese texture and flavour; (4) The extra whey proteins inhibit the melting of cheese, which is detrimental to the functionality of Mozzarella cheese; (5) Cheese ripening proceeds more slowly in the presence of extra whey proteins, because the concentration of casein is reduced, diffusion of enzymes may be hindered and β-lactoglobulin inhibits plasmin activity. Cheese manufacture from ultrafiltered milk has been most successful for fresh, low pH, soft cheese varieties, where proteolysis is limited. For this purpose the milk is concentrated usually ~ 4 - 6 fold corresponding to ~ 13 - 20% protein in the ultrafiltered milk.

5.2.5 *Novel dairy products*

Protein adjustment may be an optional treatment carried out to improve the nutritional, physico-chemical and functional properties of fluid milk and other dairy products or it may be an essential step in the manufacture of novel dairy products. The manufacture of milk protein concentrate is a good example of the latter, as it involves UF and possibly diafiltration of milk to 'up' standardize protein, so that after evaporation and spray drying, a protein rich powder is obtained. The manufacture and uses of milk protein concentrate were reviewed recently (Rosenberg, 1995).

de Wit (1989) showed the potential of protein adjustment for the manufacture of novel, texturized dairy products. Heat treatment of skim milk containing added WPC solids led to the formation of gel-like structures; when the protein content was increased by 3.0, 4.5 or 8.0%, squeezable, spreadable or cutable structures were obtained. The potential utilizability of such products has apparently not received much attention yet.

6 Conclusions

Apart from blood, milk is probably the most complex fluid known to exist, which is due in no small part to the extreme heterogeneity of the protein system. This heterogeneity is well exemplified by the fact that isolated milk proteins vary greatly in their response to heating.

A large number of studies have attempted to characterize the thermal behaviour of isolated milk proteins, especially the whey proteins, yet it is difficult to condense this information into a clear picture, due to variations in methodology and heating conditions. In milk and other dairy products, the situation is even more complicated as a large number of simultaneous and interrelated reactions take place upon heating, involving both protein and non-protein constituents.

It is now recognized that protein, rather than fat, is the most valuable constituent of bovine milk, and protein standardization or adjustment could contribute to a more realistic valorization of the protein component. However, there is a lack of knowledge of the impact of protein standardization or protein adjustment on certain properties of milk, such as its freezing point, nutritional value and sensory quality, which would be expected to strongly affect the consumer acceptability. The issue of heat stability is also important because of the need for protein-altered milk to withstand required industrial heat treatments. The investigations reported in the following Chapters attempt to address some of these concerns.

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- Chapter III -

Thermal stability of skim milk with protein content standardized by addition of ultrafiltration permeate*

Introduction

Ultrafiltration (UF) permeates consist of the fraction of milk or whey capable of permeating UF membranes. Essentially, UF permeate is an aqueous solution of lactose, minerals, non-protein nitrogen, vitamins and other low molecular weight compounds. Large quantities of UF permeates are generated during the manufacture of fresh cheeses, milk protein concentrates or whey protein concentrates and permeate utilization is a problem for the dairy industry. The extraction and subsequent applications of lactose from UF permeates were described by Short (1978) but the procedures are expensive and the market for lactose is static. Other possibilities for the utilization of whey permeate (Coton, 1980; Hansen, 1988; Renner and Abd El-Salam, 1991) are limited. Clearly, it would be useful if permeate could be used directly as a food ingredient, obviating the need for processing. In this light, the addition of UF permeate to milk for protein 'downward' standardization could be an ideal utilization procedure. Preliminary studies indicate that the addition of milk UF permeate to milk for standardization does not affect greatly the sensory (Rønkilde Poulsen, 1978) or nutritional (Smith, 1995) quality. Considering that heat treatments are involved in almost all dairy processes, information on the impact of protein standardization on the heat stability of milk would also be desirable. To date, however, scarce data exist on the heat stability of protein-standardized or otherwise diluted milk.

Fox and Hearn (1978) diluted skim milk with water and observed a marked increase in heat stability at and above the pH (~ 6.9) of minimum stability, but at other pH values there was no change or a slight increase. These results were contradicted by van Boekel *et al.* (1989b) who reported that the addition of water to milk led to a large decrease in thermal stability at pH 7.0. The latter authors suggested that the addition of water did not merely result in dilution; some colloidal calcium phosphate was dissolved to restore the level of soluble calcium, altering the nature of casein micelles. It was pointed out that at pH $\geq$ 6.9, the heat stability of milk was extremely sensitive to small changes in the concentration of soluble calcium.

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Newstead *et al.* (1977) standardized the protein content of skim milk by the addition of cheese whey UF permeate or partially-demineralized acid whey UF permeate. The protein-standardized skim milk was subsequently heat-treated (120°C/120 s), spray dried and the resultant powder used to prepare recombined evaporated milk. In general, cheese whey permeate and especially demineralized lactic acid whey permeate were detrimental to the heat stability of the recombined products; this was attributed to the relatively high salt contents of the permeates. In a similar study (Newstead, 1977) it was found that standardizing the protein content of evaporated milk by the addition of skim milk UF permeate had little influence on heat stability but when the same permeate was demineralized, a substantial increase in heat stability occurred. These studies showed that in milk the concentration of salts was more important to thermal stability than that of protein.

The aim of this study was to investigate the thermal stability of skim milk, in the pH range 6.4 - 7.1, 'down'-standardized to protein contents, in the range 2.4 - 3.44% (w/w) protein, by addition of skim milk UF permeate (SMP), sweet whey UF permeate (SWP) or acid whey UF permeate (AWP).

Materials and methods

Materials

Pasteurized (72°C/15 s), bulk skim milk, obtained from a local dairy was used to prepare SMP, SWP or AWP, and the permeates used to standardized the protein content of the same skim milk as depicted in Figure III-1. The SMP was made by pilot scale UF of skim milk, using a DDS UF unit (de Danske Sukkerfabrikker, Nakshov, Denmark) with polysulphone GR6P membranes (cut off, 25 kDa) or a Carbosep UF system (SFEC Carbosep, Bollene, France) with zirconium oxide membranes (cut off, 20 kDa). Sweet whey, produced by the addition of rennet (Sigma Chemical Co., St. Louis, MO) to skim milk (0.2 g rennet L^{-1} skim milk), was ultrafiltered using the DDS UF system to produce SWP. Acid whey, made by direct acidification of skim milk to pH 4.6 with 85% lactic acid, was ultrafiltered using the DDS or Carbosep UF systems to produce AWP. In typical experiments, ~ 500 mL of permeate was collected, necessitating UF time periods of ~ 40 min for DDS UF or ~ 5 h for Carbosep UF. The UF was carried out at pressures of ~ 400 or 2100 kPa for the Carbosep or DDS UF systems, respectively. The DDS UF was

performed at ~ 25°C; the short time of UF and the use of an effective cooling system prevented the temperature from increasing. In the case of Carbosep UF, despite the use of a cooling system, the long time period required for UF caused the temperature of the UF retentate to increase to ~ 30 - 35°C towards the end of the process. The protein content of skim milk was standardized from 3.44% (w/w) to 3.2, 2.8 or 2.4% (w/w), by addition of calculated quantities of SMP, SWP or AWP.

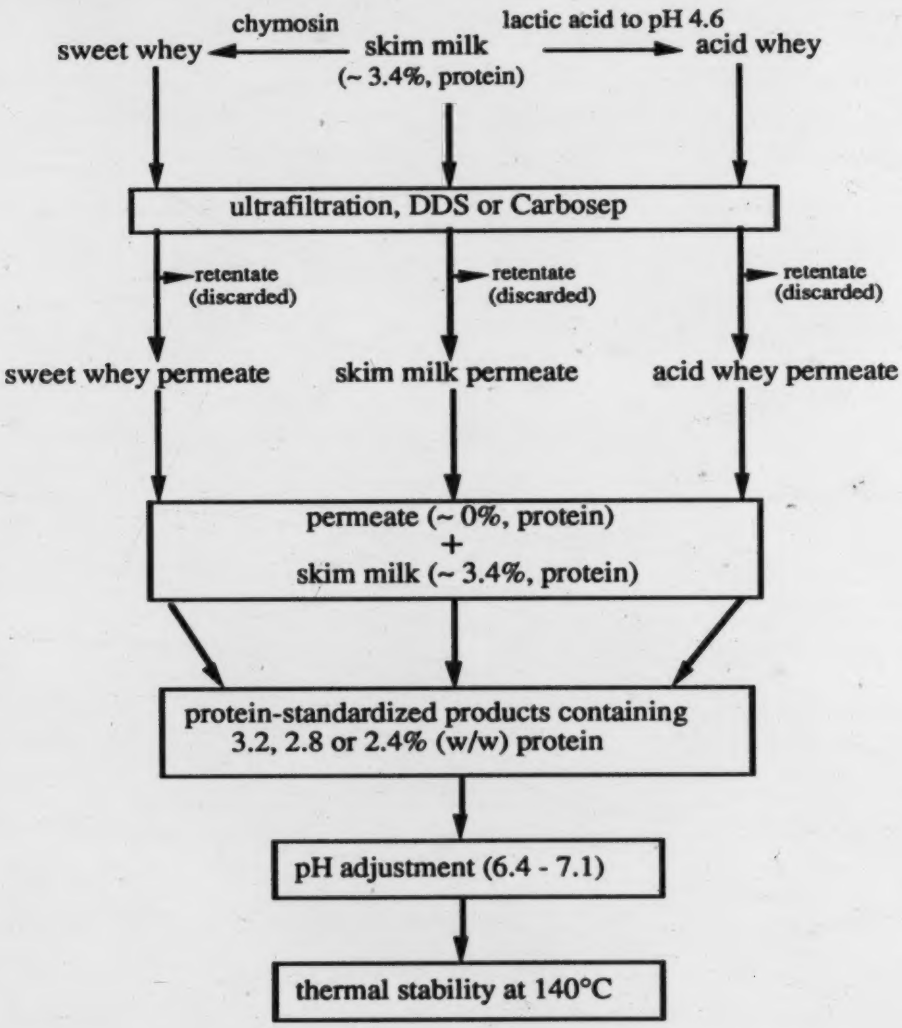


Figure III-1. Methods used to prepare protein-standardized products from skim milk.

Compositional analyses

Skim milk or UF permeates were assayed for total solids and ash by standard methods (AOAC, 1990). Total calcium was determined by atomic absorption spectroscopy of liquid permeates (AOAC, 1990). Protein was determined by the Dumas method (Grappin and Ribadeau-Dumas, 1992) using a Leco FP-428 nitrogen determination system (Leco Corp. St. Joseph, MI). Lactose was measured by an 'in house' method, developed by Food Quality Branch, Alberta Agriculture, Edmonton, Alberta, Canada; the details of the method were not disclosed.

Measurement of heat stability

Prior to determination of heat stability, the pH was adjusted to values in the range 6.4 - 7.1, by acidification from the natural pH of milk (~ 6.7) with 0.5 N lactic acid or alkalization from natural milk pH with 0.5 N NaOH. The pH was initially adjusted to the required value at 20°C, samples stored overnight at 4°C, and the pH remeasured and readjusted if necessary. Immediately after pH adjustment, heat stability was determined as the HCT, by the method of Davies and White (1966), using 140°C for most of the determinations; other temperatures from 90° to 130°C were also used where indicated. In one experiment, the pH of heat-coagulated samples was measured. For this purpose, samples just coagulated were withdrawn immediately from the oil bath, quickly cooled in a 20°C water bath and the pH determined immediately. Experiments were executed in triplicate and mean values and standard deviations calculated from all available data.

Results and discussion

Composition of skim milk and ultrafiltration permeates

The composition and pH of skim milk and UF permeates used to standardize the protein content of the skim milk are shown in Table III-1. The concentrations of ash and calcium in the SMP or SWP were considerably lower than in skim milk. The minerals of skim milk are largely associated with casein micelles (Green *et al.*, 1984); hence, removal of casein by UF or chymosin also removed most of the mineral component. However, if the entire soluble salt component of skim milk or sweet whey permeated the UF membranes, a mineral concentration of ~ 0.5 g 100 mL^{-1} would be expected (this value corresponds to the soluble salt content of milk). The fact that the concentrations of minerals in SMP or SWP

were considerably lower than this value, indicates that during UF the soluble minerals did not exhibit complete permeability. Likewise, the concentration of lactose in the SMP or SWP was slightly lower than that in skim milk, indicating incomplete permeability of lactose through UF membranes. It is possible that the low ash and lactose content of the permeates produced by Carbosep or especially DDS UF may have been due to membrane compaction and/or the relatively high pressure used during UF (Tarnawski *et al.*, 1984). The 'Donnan potential', (see section 2.3.3 of Chapter II), whereby the presence of casein micelles or whey proteins leads to increased retention of soluble ions, may have accounted for the relatively low mineral content of the permeates. Considering that in the case of UF by the Carbosep system, relatively long time periods (~ 6 h) were required to obtain sufficient quantities of permeate (~ 500 mL) for standardization of milk and the temperature of the retentate tended to increase (up to ~ 35°C) over time, it may that some fermentation of lactose occurred during UF to reduce its concentration in the Carbosep SMP. Additionally, it is possible that the tendency for the temperature of the retentate to increase towards the end of UF may have reduced the permeability of salt ions through the UF membrane and hence contributed to a relatively low concentration of salts in the SMP, due to a shift from the soluble to the colloidal state; such a phenomenon was reported by Pouliot *et al.* (1989).

Table III-1. Average composition and pH of skim milk or UF permeates made by UF of skim milk (SMP), sweet whey (SWP) or acid whey (AWP), using DDS UF or Carbosep UF systems.

Product	*pH*	*Total solids*	*Protein*[1]	*Lactose*	*Ash*	*Calcium*
		% (w/w)				*mg 100 g*$^{-1}$
Skim milk	6.7	9.3	3.44	5.1	0.70	115
SMP, DDS	6.7	3.4	0.31	2.9	0.36	21
SMP, Carbosep	6.7	5.1	0.36	4.2	0.43	27
SWP, DDS	6.7	3.4	0.30	2.8	0.35	22
AWP, DDS	4.6	3.5	0.35	2.6	0.59	62
AWP, Carbosep	4.6	5.9	0.36	4.9	0.65	83

[1]: % Protein = N x 6.38

The AWP had a relatively high calcium content; it is well known that acidification of skim milk to produce acid whey causes dissociation of colloidal calcium phosphate from the casein micelles (Holt, 1985), increasing the mineral content of the resultant whey and

hence whey permeate. The UF permeates appeared to contain ~ 0.3% (w/w) 'protein', which is typical for permeates derived by UF of milk or whey (Jacques, 1993); the 'protein' probably corresponded to the non-protein nitrogen and low molecular weight peptides capable of permeating through UF membranes. For the purposes of protein-standardization, all UF permeates were assumed to contain 0% (w/w) true protein.

The calculated amounts of permeate needed to standardize the protein content of skim milk and the resultant unadjusted pH of the protein-standardized samples are shown in Table III-2. The maximum quantity of UF permeate which could be used without a deleterious effect on heat stability, nutritional or sensory quality, would be particularly relevant. Milk products standardized with SMP or SWP had pH values (6.7) similar to the unaltered skim milk. Although AWP was rather acidic (pH 4.6), its addition to skim milk caused only a slight decrease in pH, from pH 6.7 (skim milk) to 6.6, 6.5 or 6.4 upon standardization of the protein content to 3.2, 2.8 or 2.4% (w/w), respectively, obviously due to the low buffering capacity of the protein-free permeates.

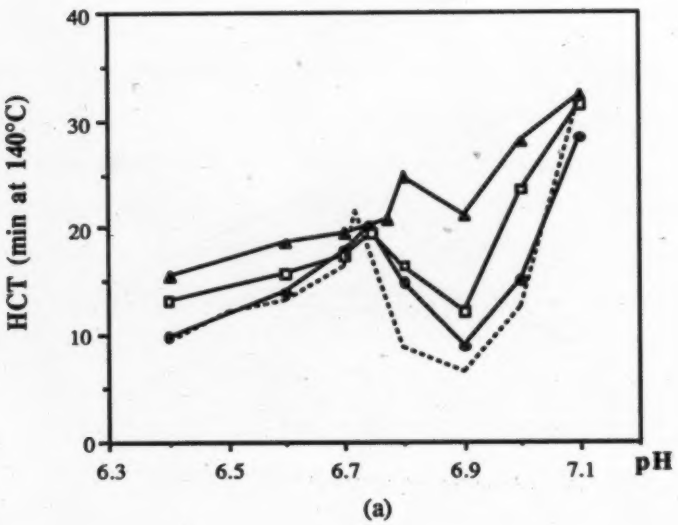
Table III-2. Calculated amounts of DDS or Carbosep UF permeates[1] added to skim milk to standardize the protein content from 3.44 to 3.2, 2.8 or 2.4% (w/w), with resultant (unadjusted) pH of the protein-standardized milk samples.

Product	*% (w/w) Protein*	*Mass of UF permeate (g 100 g^{-1} skim milk)*	*Unadjusted pH*
Skim milk	3.44	0	6.7
Skim milk + acid whey permeate	3.2	9	6.6
	2.8	25	6.5
	2.4	45	6.4

[1]: For skim milk permeate or sweet whey permeate, the mass amounts needed for protein-standardization were identical to those shown for acid whey permeate and the unadjusted pH of all samples was ~ 6.7.

Thermal stability of skim milk standardized with skim milk ultrafiltration permeate

When heated at 140°C over the pH range 6.4 - 7.1, unaltered skim milk exhibited type A behaviour. The addition of increasing amounts of SMP to skim milk caused a progressive increase in heat stability at all pH values but especially in the zone of minimum stability (Figure III-2). At the natural pH (6.7), HCT values for all samples were in the order of ~ 20 min. Despite substantial differences in total solids between the DDS and Carbosep



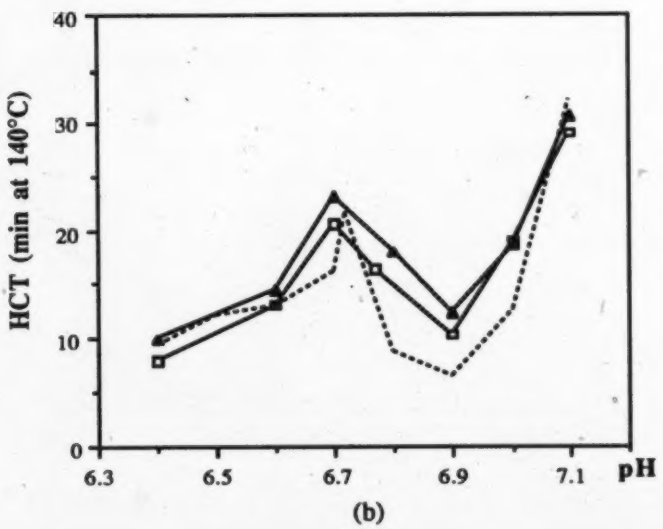


Figure III-2. Heat coagulation time (HCT) of pH-adjusted skim milk containing 3.44% (w/w) protein (----, SD = 6.4 - 18.7%) or skim milk with protein content standardized by addition of skim milk UF permeate to 3.2 (●, SD = 6.4 - 19%), 2.8 (□, SD = 7.6 - 20.1%) or 2.4 (▲, SD = 7.5 - 19.7%) % (w/w) protein. Permeates were obtained by (a) DDS UF or (b) Carbosep UF. SD indicates the range of standard deviations, expressed as percent of mean HCT values, for each type of milk.

UF permeates, there were no large differences in the heat stability responses of protein-standardized products containing either permeate. The addition of SMP to skim milk had a similar effect on heat stability compared to the dilution of skim milk with water as reported by Fox and Hearn (1978).

From compositional data (Table III-1), it is clear that adding SMP to skim milk would decrease the ratio of soluble calcium to protein. This fact may offer an explanation for the observed increases in thermal stability. The low heat stability of skim milk near pH 6.9 is probably caused by dissociation of micellar κ-casein due to its complexation with serum proteins (Kudo, 1980; Singh and Fox, 1985a, b, 1986, 1987a, b). The κ-casein-depleted micelles are very sensitive to coagulation by heat and calcium (van Boekel *et al.*, 1989a, c). Reducing the ratio of calcium to protein, by adding SMP, would not prevent the formation of κ-casein-depleted micelles, but might delay their subsequent coagulation. At pH < 6.7, the heat stability of casein micelles onto which serum proteins were deposited is relevant. While the mechanism of heat-induced aggregation of casein micelles in this region is not well understood, it is thought that calcium salts are important in the initial stages (van Boekel *et al.*, 1989a, c) and it is anticipated that milk with a reduced calcium content would show greater heat stability.

Intuitively, the dilution of protein caused by addition of SMP should also increase thermal stability by reducing the collision frequency between casein micelles. The heat stability of milk with the same salt composition but with total solids in the range 3.1 - 12.5% was studied by van Boekel *et al.* (1989b). At pH 6.6, as the concentration of total solids decreased, the HCT increased, but at pH 7.0 the total solids had no effect on heat stability. In this light, it is possible that at pH < 6.7, the increased heat stabilities of protein-standardized products were caused by a combination of dilution and changes in salt equilibria. At pH values near the minimum heat stability, it appears that thermal stability of milk is governed primarily by the salt component. However, the kinetics of casein micelle coagulation are poorly understood and the effects of dilution on coagulation rate are complex and equivocal, especially when dilution is accompanied by changes in salt equilibria (Fox and Hearn, 1978; Muir and Sweetsur, 1978; van Boekel *et al.*, 1989b). Other studies indicate that the heat stability of milk is much more sensitive to changes in the concentration of salt than of protein (Newstead, 1977; Newstead *et al.*, 1977).

In many industrial situations, the large-scale UF of milk is carried out at either ~ 4°C or ~ 50°C, in order to minimize microbial growth. The work of Pouliot *et al.* (1989) revealed a substantial effect of UF temperature on the composition of milk UF permeate; the concentrations of calcium, inorganic phosphate, magnesium or citrate declined when the temperature of UF was increased from 4 to 60°C. Considering the importance of milk salts on the heat coagulation process, it might be expected that the addition of industrially-produced permeates to skim milk would have a different effect on heat stability to that of the experimental permeates used in the present study. This issue is addressed to some extent in Chapter VI, which reports the effect of standardization with an industrial milk UF permeate on the ability of milk to withstand ultra-high temperature direct or indirect heating.

Thermal stability of skim milk standardized with sweet whey ultrafiltration permeate

Standardization of skim milk with SWP had an effect on heat stability similar to that of SMP; the HCT increased at all pH values, especially in the region of minimum heat stability (Figure III-3). Presumably, the similar compositions of SWP and SMP accounted for their equivalent effects on heat stability; the stabilizing impact of SWP can be attributed primarily to a decreased ratio of soluble calcium to protein and possibly to dilution effects.

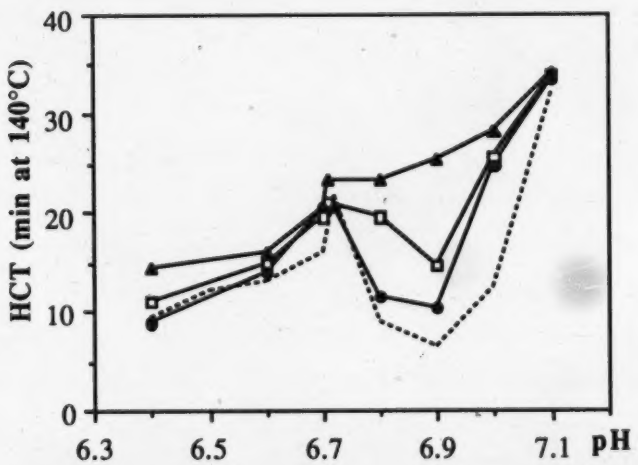


Figure III-3. Heat coagulation time (HCT) of pH-adjusted skim milk containing 3.44% (w/w) protein (----, SD = 6.4 - 18.7%) or skim milk with protein content standardized by addition of sweet whey UF permeate to 3.2 (●, SD = 5.3 - 16.9%), 2.8 (□, SD = 6.0 - 14%) or 2.4 (▲, SD = 7.2 - 19.2%) % (w/w) protein. Permeate was obtained by DDS UF. SD indicates the range of standard deviations, expressed as percent of mean HCT values, for each type of milk.

Thermal stability of skim milk standardized with acid whey ultrafiltration permeate

Addition of AWP to skim milk caused a sharp decrease in heat stability at all pH values, except near pH 6.8 where the opposite occurred (Figure III-4). Effectively, the entire HCT-pH curve was shifted to more alkaline pH values. Because a similar effect was caused by the direct addition of $CaCl_2$ to skim milk (Sweetsur and Muir, 1980), it is likely that the heat stability response induced by AWP was related predominantly to its high calcium content. Although unaltered skim milk had a greater total calcium content than AWP (Table III-1), most of this calcium (~ 2/3) exists in the colloidal state (Holt, 1985). Therefore, the addition of AWP would decrease slightly the total concentration of calcium but increase the ratio of soluble calcium to protein.

The increased heat stability of protein-standardized milk at pH 6.8 suggests that the dissociation of κ-casein from casein micelles was shifted to more alkaline pH values. Singh and Fox (1987a) proposed that near pH 6.9 a high content of soluble calcium could slow down the dissociation of soluble β-lg/κ-casein complexes from casein micelles, by competing against κ-casein as a counterion to β-lg. Therefore, it is conceivable that the addition of AWP increased calcium ion activity to such an extent that β-lg/κ-casein complexes remained on the surface of casein micelles at higher pH values. A lower rate of formation of κ-casein-depleted micelles could explain the longer heat coagulation times for milk at pH 6.8 with 3.2 or 2.8% (w/w) protein. At pH ≥ 7.0, an increasingly negative charge meant that high calcium could no longer prevent the formation of soluble β-lg/κ-casein complexes. Thus, depleted micelles may have formed and then coagulated rapidly at the high concentration of soluble calcium.

For protein-standardized milk, below initial pH ~ 6.8, it is likely that during heating serum proteins complexed onto the surface of casein micelles. It is thought that these complexes are also sensitive to calcium and that calcium may be a destabilizing factor by virtue of neutralizing negative groups on the surface of casein micelles (Pyne, 1958; van Boekel *et al.*, 1989a-c). From pH 6.4 - 6.6, the very low heat stabilities of protein-standardized products suggest that calcium effectively shifted the isoelectric pH of casein to a higher value rendering the casein more susceptible to thermal coagulation.

Another possible way by which an increased level of soluble calcium could reduce the heat stability of milk is related to the decline in pH on heating milk at high temperature. This is attributed to three changes: the decomposition of lactose to organic acids;

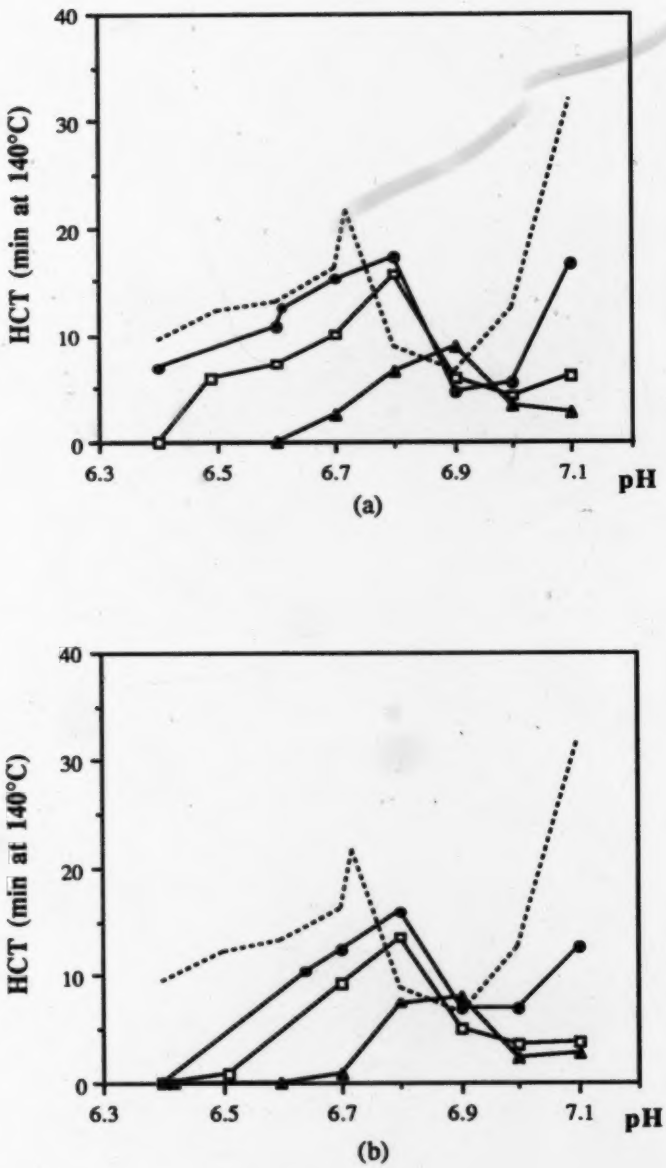


Figure III-4. Heat coagulation time (HCT) of pH-adjusted skim milk with 3.44% (w/w) protein (----, SD = 6.4 - 18.7%) or skim milk with protein content standardized by addition of acid whey UF permeate to 3.2 (●, SD = 11.3 - 16.8%), 2.8 (□, SD = 13.3 - 18.8%) or 2.4 (▲, SD = 13.8 - 21.4%) % (w/w) protein. Permeates were obtained by (a) DDS UF or (b) Carbosep UF. SD indicates the range of standard deviations, expressed as percent of mean HCT values, for each type of milk.

precipitation of primary calcium phosphate as secondary and tertiary calcium phosphates with a concomitant release of H^+ ions; and hydrolysis of ester phosphate groups of casein (Fox, 1982; van Boekel *et al.*, 1989a; Singh and Creamer, 1992; see also section 4.2, Chapter II). Increased acidity plays a vital role in the heat coagulation of casein micelles; periodic neutralization of milk at 140°C imparts extremely high thermal stability (> 3 h at 140°C) (Pyne, 1958; Fox, 1981). To determine whether the presence of AWP in milk led to a faster pH decline on heating, the pH of unaltered skim milk or skim milk standardized with AWP were measured at the HCT (after rapidly cooling the samples to 20°C) and the average rate of pH decline calculated (Table III-3). Although the pH was not measured at 140°C, the results still show a good correlation between reduced heat stability and a faster rate of pH decline. Milk standardized with AWP had faster rates of pH decline than unaltered skim milk, especially when thermal stability was very low. Within the limits of this study, an explanation cannot be offered for the enhanced rate of pH decline. One possibility is that an increased level of soluble calcium might lead to greater precipitation of tertiary calcium phosphate and hence faster release of H^+ ions.

The unadjusted pH of protein-standardized milk samples decreased slightly when standardization was carried out with AWP (with a pH of 4.6). For example, on addition of ~ 45 g of AWP to 100 g of skim milk, a sample with 2.4% (w/w) protein was obtained with the pH decreasing only from 6.7 to 6.4 (Table III-2); however, heat stability was drastically reduced. This is illustrated by plotting HCT, measured at different temperatures, against the unadjusted pH of samples standardized to various protein levels (Figure III-5). Standardization to 2.7% (w/w) protein (which reduced the pH to 6.46) led to almost instant coagulation at temperatures in the range 120° to 140°C, while at 2.4% (w/w) protein (pH 6.35) samples were unstable at temperatures as low as 90°C. The low heat stability of milk samples at unadjusted pH was probably due to the combination of low initial pH and increased soluble calcium; neutralization of negatively-charged groups caused the proteins to behave as if close to the isoelectric point. Furthermore, this destabilizing effect may have been accentuated by a more rapid rate of pH decline; for example, the heat labile milk containing 2.4% protein had an unadjusted pH of 6.4 and experienced a pH decline of 0.3 pH units min^{-1} (Table III-3).

Table III-3. Heat coagulation time (HCT)[1], pH at coagulation[1] and average rate of pH drop ($\Delta pH/\Delta t$)[1] for skim milk or skim milk standardized with acid whey UF permeate (AWP), the AWP produced using the DDS UF system.

Initial pH	*Skim milk (3.44% protein)*			*Skim milk + AWP (2.8% protein)*			*Skim milk + AWP (2.4% protein)*		
	HCT (min at 140°)	*pH at coagulation*	*$\Delta pH/\Delta t$ (min^{-1})*	*HCT (min at 140°C)*	*pH at coagulation*	*$\Delta pH/\Delta t$ (min^{-1})*	*HCT (min at 140°C)*	*pH at coagulation*	*$\Delta pH/\Delta t$ (min^{-1})*
6.4	5.3±1.0	6.14±0.1	0.05±0.02	0.8±0.3	6.20±0.1	0.25±0.07	0.6±0.1	6.22±0.4	0.3±0.06
6.6	13.3±1.1	5.87±0.2	0.05±0.02	6.2±0.9	6.15±0.1	0.07±0.02	1.8±0.4	6.27±0.3	0.18±0.04
6.7	20.2±2.8	5.69±0.1	0.05±0.02	11.2±2.1	5.99±0.3	0.06±0.01	4.7±1.2	6.30±0.2	0.09±0.02
6.8	10.1±2.0	6.13±0.1	0.07±0.02	16.5±2.2	5.81±0.2	0.06±0.02	9.3±1.3	6.00±0.1	0.09±0.02
6.9	8.1±0.9	6.27±0.2	0.08±0.03	6.5±1.1	6.32±0.2	0.09±0.02	11.1±1.6	5.97±0.1	0.08±0.01
7.0	14.0±0.9	5.77±0.2	0.09±0.03	5.5±1.0	6.35±0.2	0.12±0.04	3.2±0.6	6.51±0.2	0.15±0.03
7.1	30.5±1.7	5.49±0.1	0.05±0.01	5.4±0.8	6.58±0.2	0.10±0.03	2.8±0.6	6.70±0.2	0.14±0.02

1: Means ± standard deviation (n = 3).

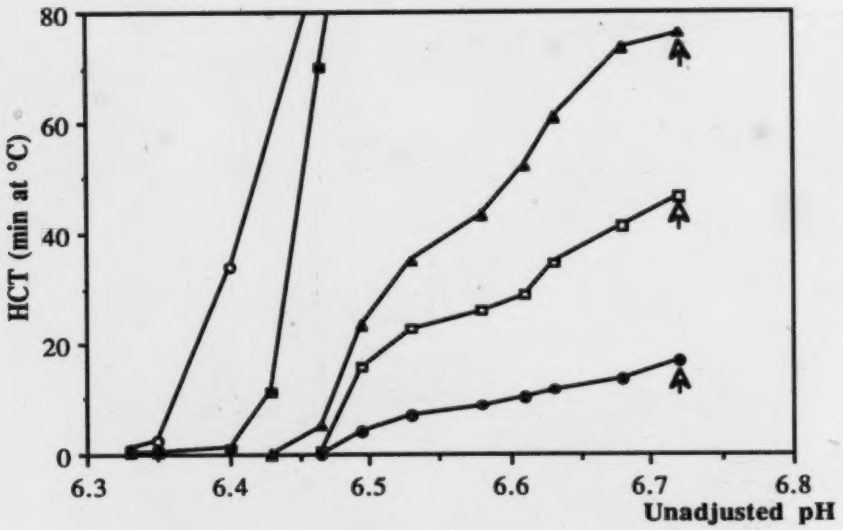


Figure III-5. Heat coagulation time (HCT), measured at unadjusted pH and at 140 (●, SD = 4.4 - 22%), 130 (□, SD = 5.6 - 20%), 120 (▲, SD = 7.5 - 23.1%), 100 (■, SD = 10.1 - 30.3%) or 90°C (○, SD =.0 - 33.6%), of skim milk with protein content standardized to different values by the addition of acid whey UF permeate, the permeate obtained by DDS UF. Arrows indicate the heat stability of unaltered skim milk (3.44%, w/w, protein; pH 6.7). Lines exiting the graph indicate HCT values in excess of 80 min. SD indicates the range of standard deviations, expressed as percent of mean HCT values, for each type of milk.

Conclusions

The heat stability of protein-standardized skim milk was unimpaired when standardization was carried out with SMP or SWP. Over the pH range 6.4 - 7.1, addition of these permeates increased heat stability and caused no appreciable changes in the natural pH of skim milk. The protein-standardized samples tended to exhibit maximum heat stability (~ 20 - 25 min) near the unadjusted pH (6.7). The use of AWP as a standardizing agent drastically reduced the heat stability of skim milk at most pH values. Although AWP caused only minor changes in the natural pH of skim milk, at natural pH, some samples were unstable at temperatures as low as 90°C. An exception was the enhanced thermal stability of skim milk standardized with AWP observed at pH 6.8. In view of the severe heating conditions used (140°C), the thermal stability of milk standardized with AWP may still be suitable for some dairy processing operations.

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- Chapter IV -

Freezing point and sensory quality of skim milk as affected by addition of ultrafiltration permeates for protein standardization*

Introduction

In the previous Chapter, detailed information on the impact of protein 'downward' standardization on the heat stability of milk was presented. While such information is useful in assessing the heat processability of protein-standardized milk, other issues must also be addressed if protein standardization is to become a widespread practice. These include the impact of standardization on the sensory quality of milk, especially important if the milk is for direct consumption, and the effect of standardization on the freezing point (FP) of milk, which might have implications from a regulatory viewpoint.

Rønkilde Poulsen (1978) standardized the protein content of skim milk, 1.8% fat milk or 3.5% fat milk, in the range 1.5 - 6.5% protein, using reverse osmosis (RO) or ultrafiltration (UF) procedures. When the protein content of milk was altered by RO, the concentrations of lactose and soluble salts changed also and this had a strong influence on the sensory quality; at ± 0.3% SNF the milks elicited either watery or salty/sweet sensations. In contrast, protein-standardization by removal or addition of milk UF permeate caused lesser changes in the concentrations of lactose or soluble salts, making distinction between normal milk and protein-standardized milk very difficult, especially as the fat content increased; for skim milk, panelists could not detect differences between samples with 3.1 - 6.5% protein, while protein-standardized whole milk samples were mutually indistinguishable when protein concentration was in the range 1.5 - 6.4%. Milk standardized to a low protein concentration was identified by a translucent appearance rather than an altered taste.

The FP of protein-standardized milk could be important, because if the FP would be altered, protein-standardized milk could be considered as adulterated or otherwise abnormal. Although the interpretation of FP data can vary due to slight regional and seasonal variations and milk handling factors (van der Berg, 1979; Eisses and Zee, 1980; Sherbon, 1988; Schukker *et al.*, 1992; Covney, 1993), in general, normal milk freezes near - 0.522°C, while a FP > - 0.508°C is considered to be a definite proof of extraneous

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water (AOAC, 1990). It should be noted that in much of the literature, FP data were expressed incorrectly as °C, when °H should have been used (Sherbon, 1988). Because the addition of UF permeate is likely to cause small changes in the concentrations of lactose and minerals, as well as protein, changes in the FP may be anticipated.

In the present study, the impacts of protein 'down' standardization using four types of UF permeate on the sensory quality and FP of skim milk were determined. A regular market skim milk was standardized by adding skim milk UF permeate (SMP), sweet whey UF permeate (SWP) or permeates obtained by UF of whey made by direct acidification of skim milk to pH 4.6 (AWP) or by fermentation of skim milk to pH 4.6 with lactic acid bacteria (FWP).

Materials and methods

Materials

The SMP, SWP and AWP were made by procedures identical to those described in Chapter III, except that all permeates were made using the Carbosep UF system only. In addition, a second type of acid whey permeate was made by the UF of whey from a fermented dairy product, denoted as 'FWP', as opposed to the 'AWP' which was made by UF of directly acidified milk. Fermentation of skim milk by lactic acid bacteria to pH 4.7 yielded whey for the FWP. The fermentation was carried out at 35°C for 20 h using a mixed culture of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, used normally in the manufacture of sour cream by a local dairy company. The protein content of skim milk was standardized to values in the range 3.44% to 2.1% (w/w) by adding calculated quantities of SMP, SWP, AWP or FWP or in some cases using blends of these UF permeates.

Compositional analyses and measurement of freezing point

The compositions of skim milk and the UF permeates were determined by standard methods (AOAC, 1990). The FP data of UF permeates, normal milk or protein-standardized milk were obtained in triplicate by the Hortvet method (AOAC, 1990), using a Milk Cryoscope (Advanced Instruments Inc., MA). The principle of the method used to determine FP was described in section 2.4.2 of Chapter II. The cyroscopic FP data were converted from °H to °C according to a formula developed by AOAC (1990).

Assessment of sensory quality

The sensory quality of protein-standardized skim milk was evaluated by an untrained panel of 15 individuals selected at the University of Alberta, using the triangle test method (Jellinek, 1985). Skim milk was especially suitable for sensory evaluation of the effects of UF permeate, as the absence of milk fat increases the sensitivity of individuals to changes in lactose and/or salts (Rønkilde Poulsen, 1978). Milk samples (~ 30 mL) were served at 15°C ± 1°C in small plastic containers, which were randomly numbered using a two digit code. In isolation, each panelist was presented with three samples and asked to identify the odd sample on the basis of appearance, taste and odour, guessing if necessary. The odd sample consisted of either protein-standardized skim milk or normal skim milk. Triangle test results were analyzed statistically using tables developed by Roessler *et al.* (1978).

Results and discussion

Freezing point of protein-standardized milk

Compared to normal skim milk, SMP or SWP had relatively high FP values, attributed to their lower lactose and salt contents (Table IV-1). The relatively low ash and lactose contents of SMP or SWP, also observed in Chapter III, were due to the removal of casein from skim milk by UF or chymosin, respectively, and incomplete permeability of soluble minerals and lactose through the UF membranes used. Because colloidal minerals exist as part of the casein micelle, a high molecular weight entity, their removal from milk or sweet whey would be expected not to affect the FP. Therefore, the higher FP values of the SMP or SWP, compared to the FP of skim milk, must have been caused by relatively low concentrations of soluble salts and lactose in the permeates.

The relatively high salt contents and titratable acidities of the AWP or FWP probably contributed to their low FP values. Clearly, the high titratable acidity of these permeates was caused by direct addition of lactic acid to skim milk, involved in the production of whey for the AWP, or by fermentation of lactose to lactic acid, during the manufacture of whey for the FWP. The high salt contents of the AWP or FWP can be attributed to dissolution of colloidal calcium phosphate from casein micelles upon acidification (Holt, 1985). It is interesting to note that the FP of the FWP was slightly lower than that of the AWP. This may have been caused by some degradation of protein into peptides and free amino acids by lactic acid bacteria, causing the molality of the FWP

to be increased. The addition of AWP or FWP to skim milk was expected to increase the level of dissolved salts and lactic acid, thus reducing the FP of protein-standardized milk, as was indeed observed (Figure IV-1).

Table IV-1. Average composition (%,w/w), pH and freezing point (°C) of skim milk or ultrafiltration (UF) permeate obtained from skim milk (SMP), sweet whey (SWP), acid whey produced by direct acidification of skim milk (AWP) or acid whey produced by fermentation of skim milk (FWP).

Product	*pH*	*Total solids*	*Protein*[1]	*Lactose*	*Ash*	*Titratable acidity*[2]	*Freezing point*
		% (w/w)					°C
Skim milk	6.7	9.5	3.44	5.0	0.70	0.168	- 0.521
SMP	6.7	5.11	0.33	4.4	0.31	0.086	- 0.465
SWP	6.7	5.19	0.31	4.4	0.32	0.119	- 0.420
AWP	4.6	5.90	0.36	4.3	0.57	0.511	- 0.593
FWP	4.7	5.58	0.29	4.1	0.64	0.502	- 0.610

[1]: % Protein = % N x 6.38

[2]: Expressed as % (w/w) lactate

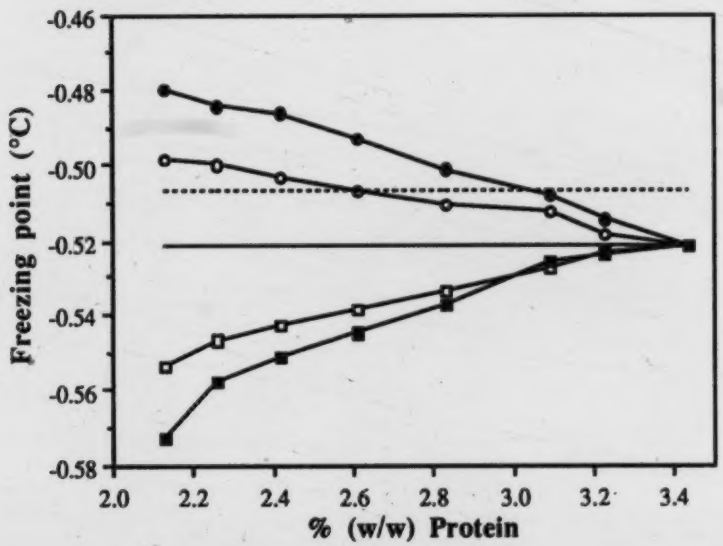

Figure IV-1. Freezing point of normal skim milk (3.44%, w/w, protein; —, SD = 0.40%) or skim milk with protein content standardized to values in the range 3.44% - 2.1% (w/w) protein, by addition of skim milk UF permeate (○, SD = 0.28 - 1.3%), sweet whey UF permeate (●, SD = 0.47 - 1.61%), acid whey UF permeate (□, SD = 0.66 - 2.01%), fermented whey UF permeate (■, SD = 1.1 - 3.3%). A freezing point > - 0.508°C (---) is conventionally regarded as proof of the presence of extraneous water. SD indicates the range of standard deviations, expressed as percent of mean FP values, for each type of milk.

It has been recommended that the FP of milk should not exceed - 0.508°C; otherwise, the milk is deemed to contain extraneous water (AOAC, 1990). In this light, milk standardized with SMP to ≤ 2.6 or SWP to ≤ 3.1% protein could be considered as adulterated (Figure IV-1). The decreased FP of milk standardized with AWP or FWP suggests also a noticeable change in composition, indicative of abnormal milk. Because the addition of UF permeates to skim milk leads to only small alterations in nutritional (Smith, 1995; also see Chapter VI) or sensory quality (Rønkilde Poulsen, 1978; also see below), it could be argued that designating protein-standardized milk as 'adulterated' or otherwise abnormal would be unjustified or at least that the addition of UF permeate to milk should not be equated with the addition of water. However, it is still possible that an altered FP would reduce the acceptability of protein-standardized milk.

In view of the present results, were protein standardization of fluid milk to become widespread, measurement of FP should be carried out prior to the addition of UF permeate, to avoid erroneous interpretation of FP data. In practice, this is likely to occur, as protein standardization almost certainly would be carried out on bulk milk and FP measurements are usually used to test the quality of milk from individual farmers, rather that bulk milk.

If protein-standardized milk had the same FP as normal milk, equivocal interpretation of FP data could be avoided. It was observed that the increasing addition of AWP to either SMP or SWP caused the FP to increase gradually (Figure IV-2), presumably due to an increasing content of salt and lactic acid. The AWP in combination with either SMP (40/60) or SWP (60/40) had a FP close to - 0.521°C, which was the same as normal skim milk. Thus, by adding these particular combinations of UF permeate to skim milk, the protein content could be standardized 'down' to at least 2.1% (w/w) with negligible changes in FP (Figure IV-3). Similarly, appropriate ratios of FWP to either SMP (40/60) or SWP (50/50) were established.

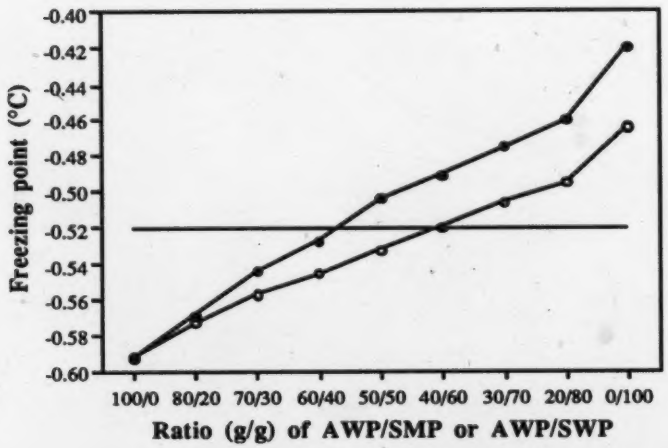

Figure IV-2. Freezing point of skim milk (—, SD = 0.40%), combinations of acid whey permeate and skim milk permeate (AWP/SMP) (●, SD = 0.68 - 1.1%) or combinations of acid whey permeate and sweet whey permeate (AWP/SWP) (○, SD = 0.77 - 0.98%). SD indicates the range of standard deviations, expressed as percent of mean FP values, for each type of milk.

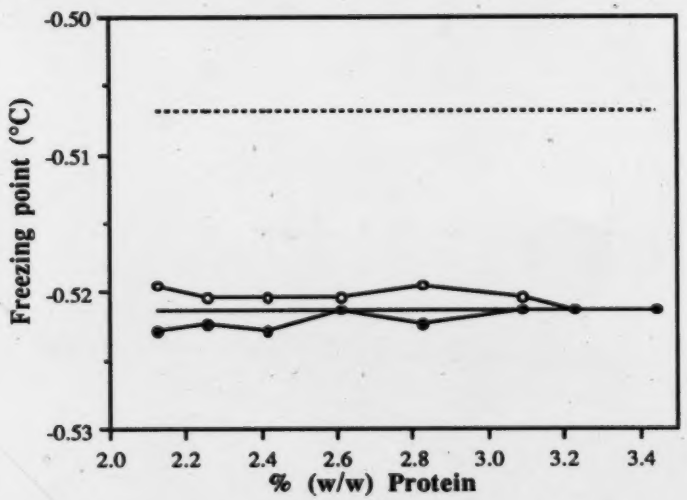

Figure IV-3. Freezing point of normal skim milk (3.44%, w/w, protein; —, SD = 0.40%) or skim milk with protein content standardized to values in the range 3.44% - 2.1% (w/w) protein, by addition of a 40/60 ratio of acid whey permeate/skim milk permeate (●, SD = 0.51 - 0.88%) or a 60/40 ratio of acid whey permeate/sweet whey permeate (○, SD = 0.56 - 1.0%). A freezing point > - 0.508°C (---) is conventionally regarded as proof of the presence of extraneous water. SD indicates the range of standard deviations, expressed as percent of mean FP values, for each type of milk.

Sensory quality of protein-standardized milk

The sensory impact of bovine milk, as an interaction of taste, odour and texture, is a very complex phenomenon, especially in the presence of milk fat. In the absence of milk fat, the taste is influenced primarily by lactose and salts with protein having a relatively minor effect (Pangborn and Dunkley, 1966). An exceedingly complex 'cocktail' of compounds contributes to the odour of milk; Badings and Neeter (1980) identified approximately 400 volatile compounds in whole milk, but it was not possible to ascribe a milk-like odour to any one compound or restricted group of compounds. It appears likely that if protein standardization involved exclusive changes in protein, the taste or odour would not be altered significantly.

In sensory evaluation trials, protein-standardized milk products containing SMP or SWP were difficult to distinguish from unaltered skim milk. Standardization was possible to ≤ 2.4% (w/w) protein before significant differences occurred (Table IV-2). In agreement with Rønkilde Poulsen (1978), milk with a low protein content was detected due to a slightly translucent appearance, rather than any noticeable change in taste or odour. As reported in Chapter III, protein-standardized and normal skim milk had equal pH values (pH 6.7) which may have contributed to their indistinguishability. Clearly, the increased translucency evident at ≤ 2.4% protein resulted from a lower concentration of casein micelles which reduced the ability of skim milk to scatter light. Although decreases in the content of lactose and soluble salts would be expected to occur upon addition of permeates, as evident by compositional data (Table IV-1) and the altered FP of protein-standardized milk (Figure IV-1), these were insufficient to be detected by the taste panel. In practice, it is unlikely that milk would be standardized down to a values as low as 2.4% (w/w) protein; therefore, industrial standardization with SMP or SWP would not reduce the sensory acceptability.

Standardization of skim milk with AWP was possible down to 2.8% (w/w) protein, before a substantial alteration of sensory quality occurred (Table IV-2). At 2.8% (w/w) protein, the presence of AWP was just perceptible; most participants reported that milk had a salty and slightly acidic taste, probably caused by increased concentrations of dissolved salts and lactic acid; notably, the pH of skim milk declined slightly upon addition of AWP. However, standardization to ≥ 3.0% (w/w) protein, produced milk that was essentially

indistinguishable from normal skim milk, indicating that AWP could be suitable also for a limited degree of protein standardization.

Table IV-2. Sensory differences between normal skim milk (3.44%, w/w, protein; pH 6.7) and skim milk standardized to 3.2 - 2.4% (w/w) protein, by addition of UF permeate, determined by triangle tests involving 15 individuals. Permeates were made by UF of skim milk (SMP), sweet whey (SWP) or acid whey produced by direct acidification of skim milk to pH 4.6 (AWP) or by fermenting skim milk to pH 4.7 (FWP).

Permeate type for standardization	*% (w/w) Protein of standardized skim milk*[1]	*pH of protein-standardized skim milk*	*Number of correct results*[2]
SMP	3.2	6.7	4 (n.s.)
	2.8	6.7	5 (n.s.)
	2.4	6.7	9 (*)
SWP	3.2	6.7	4 (n.s.)
	2.8	6.7	6 (n.s.)
	2.4	6.7	10 (**)
AWP	3.2	6.6	3 (n.s.)
	3.0	6.6	3 (n.s.)
	2.8	6.5	9 (*)
	2.4	6.4	13 (***)
FWP	3.2	6.6	12 (***)
	3.0	6.6	12 (***)
	2.8	6.4	14 (***)
AWP/SMP (40/60)	2.8	6.6	4 (n.s)
	2.4	6.5	10 (**)
FWP/SMP (40/60)	3.2	6.7	6 (n.s.)
	3.0	6.7	9 (*)

[1]: Standardization was on the basis that all permeates contained 0% true protein; skim milk (3.44%, w/w, protein) was standardized to 3.2, 3.0, 2.8 or 2.4% (w/w) protein, by addition of 7.5, 15, 23 or 43 g of UF permeate per 100 g of skim milk, respectively.
[2]: No significant difference (n.s.) or significant differences between normal skim milk and protein-standardized skim milk for $\alpha < 0.05$, 0.01 or 0.001, as indicated by (*), (**) or (***), respectively.

The FWP was unsuitable as a protein standardizing agent. Even at 3.2% (w/w) protein, where the pH change was negligible, a strong off-flavour in the milk was evident by olfaction or gustation. Doubtless, the off-flavour was caused by the presence of low molecular weight compounds, originating from the fermentation of lactose. The principal

volatile compounds produced by lactic acid bacteria are diacetyl, acetaldehyde, dimethyl sulphoxide, acetic acid and lactic acid (Lindsay *et al.*, 1967), all of which should easily pass through the UF membrane used. The exact compound(s) responsible for the presence of the off-flavour in the protein-standardized milk were not determined in this study. It is probable that the nature and extent of off-flavour development would depend on the type of micro-organisms used and the fermentation conditions. In the present study, the use of a sour cream culture could be expected to produce relatively high amounts of flavourful compounds.

As shown above, it was established that appropriate combinations of UF permeates might be especially suitable for protein standardization, if it was desired to obtain protein-standardized milk with an unaltered FP. To test further the suitability of these blends for standardization, the sensory quality of normal skim milk was compared to skim milk standardized with the appropriate UF permeate combinations. The results indicated that the sensory quality of milk products standardized with mixtures of AWP and SMP (40%/60%) was satisfactory (Table IV-2), which was not surprising since the use of either permeate alone was also satisfactory. Moreover, the addition of SMP to AWP extended the limit of detectability to ≤ 2.8% (w/w) protein, compared to the use of AWP alone, possibly due to smaller changes in pH, and the concentrations of salts and/or lactic acid. Although FWP/SMP combinations were less deleterious to the sensory quality than FWP alone, these mixtures still appeared unsuitable for industrial standardization as milk with even 3.0% (w/w) protein possessed a noticeable off-flavour.

Conclusions

SMP or SWP appear to be suitable materials for standardizing the protein content of skim milk, provided the resultant increase in FP is not equated with adulteration; such an interpretation may not be justified as changes in sensory quality were insignificant at > 2.4% (w/w) protein and nutritional changes are also considered to be unimportant. Triangle test results suggest also the suitability of AWP for protein standardization; sensory changes were insignificant at > 2.8% (w/w) protein; however, the substantially decreased FP of milk standardized with AWP could lead to such milk being designated as abnormal. If the alteration of the FP of milk were to be avoided during standardization, then a possible solution might be to standardize milk with an appropriate combination of UF permeates,

which caused no change in the FP and sensory properties also appeared acceptable. The use of FWP for protein standardization was unacceptable; apart from possible problems associated with a lower FP of protein-standardized milk, a strong off-flavour occurred, evident even for very low additions of FWP to skim milk.

It should be emphasized that the four kinds of UF permeate used in this study were experimental by-products obtained from a limited number of UF trials using skim milk as the primary raw material. Many types of whey are produced industrially, due primarily to the diversity of cheese manufacture (Sienkiewicz and Riedel, 1990). The composition of UF permeates and hence their suitability for protein standardization will depend both on the type of whey and conditions during the subsequent UF process. Moreover, as noted in Chapter III, the mineral composition of milk UF permeate is strongly affected by the temperature of UF. Thus, the data presented in this study give only an illustration of the issues likely to be involved in determining the suitability of different UF permeate types for protein standardization.

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- Chapter V -

Heat stability and sensory quality of protein-standardized 2% fat milk*

Introduction

In the previous two Chapters, investigations of the suitability of various ultrafiltration (UF) permeate types for the 'downward' standardization of milk protein were discussed. These studies involved the standardization of skim milk, rather than fat-containing milk, because of the ease of measuring heat stability in skim milk and also because the absence of milk fat was anticipated to assist in the sensory discrimination between normal and protein-standardized milk. While skim milk constitutes an important dairy product, it is still true that milk with fat is even more important, especially when the milk is for direct consumption. Furthermore, the heat stability studies of the previous Chapters were carried out on milk which had been adjusted to various initial pH values; while this gives a more complete view of heat stability phenomenon, industrial situations usually require that milk be heat-treated at its unaltered or natural pH. Additionally, the sensory quality or heat stability of milk with its protein content standardized 'upward' by UF or the addition of UF retentate was not investigated, even though the International Dairy Federation has also expressed interest in standardizing protein by this method (Marshall, 1995).

In section 5 of Chapter II, a distinction was made between protein adjustment and protein standardization; protein adjustment involves a much more drastic modification of the milk protein component, which may alter the ratio of whey protein to casein. Protein adjustment may be advantageous in the manufacture of cheese, cheese analogues, yoghurt and sour milk and provide nutritional benefits. Clearly, basic information on the heat stability of protein-adjusted milk would be desirable, as this would have implications with respect to its processability.

To address some of the above issues, in the present study, the protein content of 2.0% fat milk was altered from 3.44% to the range 2.0 - 4.0%, without alteration of fat content, using three relatively simple techniques, namely, by direct addition of a dried fraction of α-lactalbumin (α-la) to 2.0% fat milk; by blending calculated combinations of skim milk UF retentate (SMR), partly skimmed milk (~ 2% fat) and whole milk (~ 3.5% fat); or by blending skim milk UF permeate (SMP), partly skimmed milk and

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whole milk. Addition of α-F to milk increases the ratio of whey protein to casein protein and thus can be regarded as a form of protein adjustment, while enrichment of milk with SMR or SMP can be considered as 'up' or 'down' protein standardization, respectively. The effects of these protein manipulation treatments on heat stability was measured by the subjective heat coagulation test and, in cases where milk fat impeded the visual observation of heat coagulation, by measuring the mass of centrifugable material. In Chapter VIII, the results of a more detailed study of the protein adjustment on the heat stability of milk are presented. Additionally, the sensory quality of milk with an altered protein fraction was assessed by triangle difference tests.

Materials and methods

Materials

Skim milk, partly skimmed homogenized milk and whole homogenized milk were obtained from a local dairy company; all were commercial products, which had been pasteurized at 72°C/15 s. The α-fraction (α-F), produced on an industrial scale, was provided by Protose Separations Inc., Toronto, Ontario, Canada; the manufacturing procedure was not disclosed. The skim milk was ultrafiltered, using a Carbosep UF system, to obtain a retentate with ~ 5% protein and a permeate with ~ 0% protein, both of which were kept for protein standardization procedures. The UF was carried out using the same conditions as described in Chapter III.

Compositional analyses

The protein content of the milk was measured according to AOAC (1990). The purity of the protein fraction in the α-F was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). To this end, the α-F was dissolved in distilled/deionized water and mixed with SDS reducing buffer (an aqueous solution of 9% SDS, 15% mercaptoethanol, 30% glycerol, 0.01% bromothymol blue, 150 mM tris HCl at pH 6.8) to obtain a final protein concentration of 2.0 mg mL^{-1}. A 12.5% polyacrylamide gel was then injected with 5 μL of the protein solution and the proteins separated by application of an electrical potential of 75 V for 20 min, followed by 150 V for 1 h. After separation the gel was stained with Coomassie Blue for ~ 12 h and then destained with a water-methanol-

acetic acid solution. Species of protein were readily identified on the basis that, in the presence of SDS, electrophoretic motility would be proportional to molecular weight.

Modification of the protein fraction of milk

In this study, control milk consisted of a regular 2% fat homogenized milk, which contained 3.44% (w/w) protein. The protein content of 2% fat milk was 'up'-standardized by mixing calculated quantities of partly skimmed milk, whole milk and SMR to obtain milk samples with 2.0% (w/w) fat and 3.44 - 4.0% (w/w) protein. The 'down' standardization of 2% fat milk was accomplished by mixing required amounts of partly skimmed milk, whole milk and SMP to obtain milk products with 2.0% (w/w) fat and 2.0 - 3.44% (w/w) protein. The direct addition of dried α-F to 2% fat milk increased the protein content in the range 3.44 - 4.0% (w/w). The quantities of α-F added were so small (0 - 0.67 g to 100 g milk) that variations in the fat content of the milk were calculated to be negligible (1.98 - 2.0%).

Measurement of heat stability

The heat stabilities of all milk samples were measured by the subjective heat stability test, as used also in Chapter III. In some cases, the presence of milk fat obscured the coagulation point so that visual determination of heat stability was imprecise and a second technique was needed to more rigorously establish heat stability trends. In this method, milk samples were heated under the same conditions as for the subjective HCT test for 0, 5, 10, 15, 20 or 25 min, withdrawn and immediately cooled to 20°C. A sample (15 mL) of heated milk was centrifuged at 900 x g for 20 min, using an IEC HN-SII centrifuge (Damon/IEC Division, Needham Heights MA) and heat stability assessed by measuring the mass of centrifugable material obtained for different times of heating at 140°C. The centrifugation tests and the visual determination of heat stability were executed in duplicate and quadruplicate, respectively, from which mean heat stabilities and standard deviations were calculated. All heat stabilities were measured at the unaltered pH of the milk samples, which displayed little variation (pH 6.64 - 6.69) upon alteration of the protein component by any of the methods used.

Sensory quality

The sensory effects of protein standardization were determined by comparing the protein-standardized or protein-adjusted milk products to control milk, using triangle tests with an untrained taste panel of 17 individuals, selected from staff and students at the University of Alberta. The sensory test method was exactly the same as described in Chapter IV and the results were analyzed using tables produced by Roessler *et al.* (1978).

Results and discussion

Thermal stability of milk standardized with skim milk ultrafiltration retentate

Increasing the protein content of 2.0% fat milk, up to at least 4.0% protein, by fortification with SMR appeared to have a negligible effect on heat stability, as determined by the subjective heat stability test (Figure V-1).

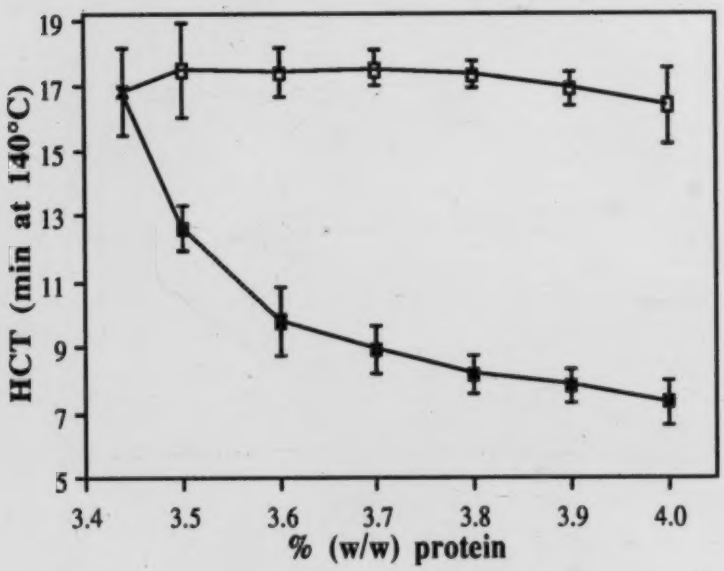

Figure V-1. Heat coagulation time (HCT) of 2% fat milk with protein content adjusted from 3.44% (control, x) up to 4.0%, by fortification with skim milk UF retentate (□) or α-fraction (■). Error bars calculated as the standard deviation of the data (n = 4).

However, it was difficult to observe the onset of protein coagulation in the presence of milk fat, either because the fat globules obscured the appearance of protein aggregates and/or caused protein aggregation to be more gradual. Thus, to more stringently establish the heat stability trend, the centrifugation test method was used. After 15 min of heating, almost twice as much sedimentation occurred in the protein-standardized milk samples (0.78 - 0.86 g) as in the control milk (0.46 g), indicating a greater degree of protein aggregation in the former (Figure V-2).

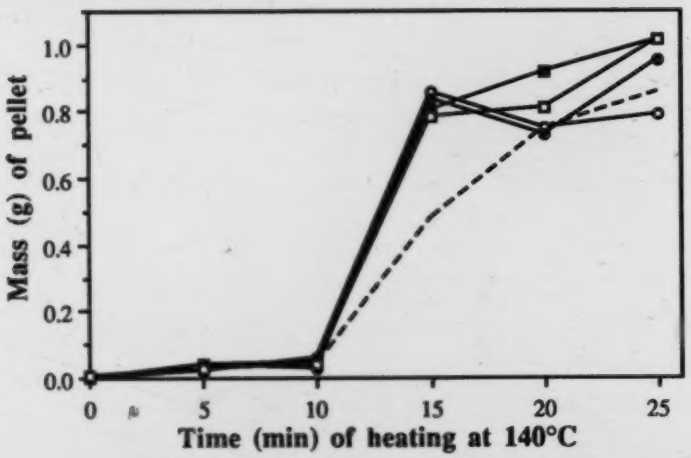

Figure V-2. Mass of pellet obtained after centrifugation (900 x g) of 15 mL of milk, heated at 140°C for 0 - 25 min. The protein content of 2% fat milk was standardized from 3.44% (control, - - -) to 3.5 (○), 3.6 (●), 3.8 (□), or 4.0 (■)% by fortification with skim milk ultrafiltration retentate. Error bars are excluded to preserve clarity.

The heat-treated, SMR-enriched milk samples, due to their greater protein content, were expected to have greater pellet masses than the control milk, but the magnitudes of the actual increases (~ 63 - 79%) were disproportionately large. This implies that the greater masses of centrifugal pellet were caused predominantly by reduced heat stabilities. Furthermore, the pellet masses of protein-standardized milk samples showed very little increase between 15 and 20 min heating, but the pellet mass of the control milk almost doubled. Therefore, protein aggregation was more gradual in the control milk, most of it occurring between 10 - 20 min of heating, whereas the protein-standardized milk products destabilized more quickly, between 10 - 15 min of heating.

Determination of heat stabilities by the HCT tests showed no difference between the control and the protein-standardized milk products, whereas the centrifugation technique did, probably because relatively small protein aggregates could be sedimented at 900 x g, compared to those which could be detected visually, at ~ 1 x g, in the subjective HCT test. The centrifugation test method was very similar in principle to the objective heat stability test described by White and Davies (1966), which measures the percentage of total nitrogen remaining soluble after low speed centrifugation of heated milk. These authors opinionated that the subjective heat stability test was only suitable when protein coagulation was discrete; for more gradual heat aggregation processes, the objective heat stability test was favoured. A disadvantage of the objective heat stability test is that heat stability is measured after cooling to room temperature, rather than at the temperature of heating.

During the UF of skim milk, the UF membrane retains protein but allows water, lactose and salts to permeate to increase the ratio of total protein to water, lactose and salts in the retentate and thus in milk containing added UF retentate. An increased concentration of total protein would increase the rate of intermolecular collisions during heating, which would tend to reduce heat stability (van Boekel *et al.*, 1989b), but the slightly lower levels of dissolved salts and lactose might compensate partially for this effect and cause the reduction in heat stability to be less drastic. The results here indicate that 'up' standardization by UF of the milk caused a small decrease in heat stability, which was only detectable using the sensitive centrifugation technique. The decline of heat stability was so small that it probably would be of little industrial importance.

Thermal stability of milk with α-fraction

As seen above in Figure V-1, the addition of increasing amounts of α-F to 2.0% fat milk caused a progressive decline in heat stability. The establishment of a clear heat stability trend obviated the need to employ the more tedious centrifugation test.

The α-F contained 89.9% total protein, 4.1% moisture, 2.9% lactose and 1.0% ash. As revealed by SDS-PAGE, α-la was the most abundant protein with lesser amounts of β-lactoglobulin (β-lg) and bovine serum albumin (data not shown). At pH 6.7, α-la denatures at about 65°C (Rüegg *et al.*, 1977), but denatured α-la is extremely resistant to thermal aggregation, even in the presence of soluble milk salts (Larson and Rolleri, 1955). This may imply that the reduced heat stability of milk enriched with α-F was not caused by

independent coagulation of α-la. It is also unlikely that the extra α-la in the protein-adjusted milk products reduced the heat stability by thermal interaction with casein micelles; Fox and Hearn (1978) established that near the unaltered pH of milk, α-la was capable of thermal complexation with casein micelles which increased their charge and hydration and thus resistance to heat aggregation. Probably, the low level of β-lg in the α-F was responsible for the reduced heat stability of the α-F-enriched milk samples, considering that enrichment of milk with small amounts of β-lg causes a drastic reduction in the heat stability of casein micelles (Patocka *et al.*, 1993). However, it is not clear whether milk with extra β-lg is heat labile due to the coagulation of β-lg alone and/or due to the coagulation of β-lg-casein micelle complexes. Despite the presence of milk fat, the visual determination of the heat stability of milk with α-F was relatively easy, indicative of a sudden formation of large protein clots. This suggests that whey proteins did coagulate, either independently and/or after complexation with casein, because the thermal aggregation of casein micelles alone is thought to be a gradual process, whereas whey protein aggregation proceeds more rapidly and discretely.

The reduction of heat stability caused by the presence of the α-F was quite pronounced but even the most heat labile milk (4.0% protein, HCT ~ 7 min) would be expected to easily withstand most industrial heat treatments. The results show that whey protein products rich in α-la are suitable for altering the protein content of milk without a deleterious effect on heat stability, in contrast to milk enriched with a β-lg-rich whey protein concentrate (Patocka *et al.*, 1993).

Thermal stability of milk standardized with skim milk ultrafiltration permeate

The subjective heat stability test was extremely difficult to perform with 'down'-standardized, fat-containing milk and, despite repeated trials, reliable results could not be obtained (Figure V-3); the ability of fat to obscure the coagulation point may have been assisted by a lower concentration of protein. However, the centrifugation test showed that milk 'down'-standardized with SMP was more heat stable than unaltered milk. Whereas control 2.0% fat milk destabilized after 10 - 20 min of heating, the protein-standardized milk became unstable after 15 - 20 min heating (Figure V-4). Moreover, after 20 min of heating, the pellet masses of protein-standardized milk products (0.45 - 0.72 g) were consistently smaller than in the control milk (0.75 g). After 15 min of heating, the masses

of pellet in milk products with 2.4 - 2.0% protein were greater than in the other protein-standardized samples (but less than in control milk), probably because the lower viscosity allowed smaller protein aggregates to be sedimented.

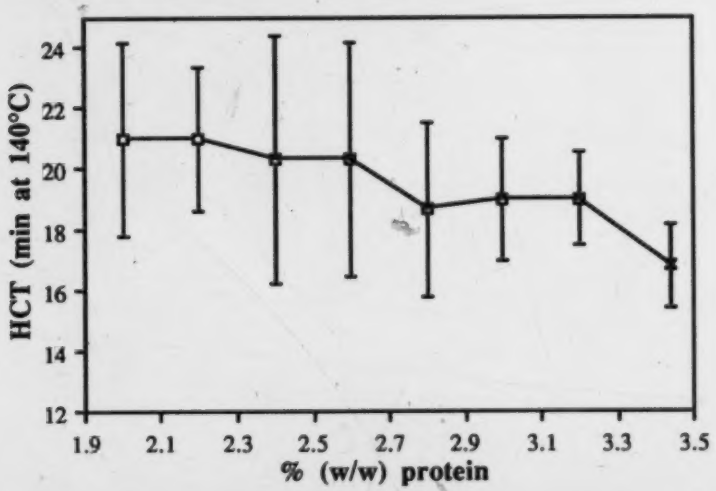

Figure V-3. Heat coagulation time (HCT) of 2% fat milk with protein content adjusted from 3.44% (control, x) down to values in the range 3.2 - 2.0% (□), by using skim milk UF permeate. Error bars calculated as the standard deviation of the data (n = 4).

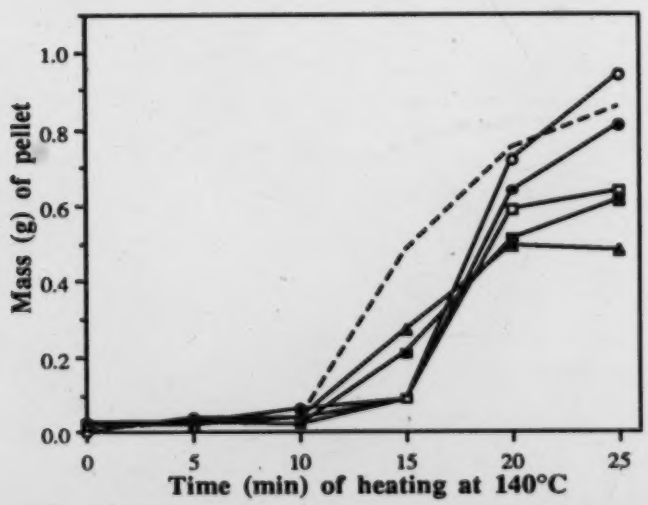

Figure V-4. Mass of pellet obtained after centrifugation (900 x g) of 15 mL of milk, heated at 140°C for 0 - 25 min. The protein content of 2% fat milk was standardized from 3.44% (control, - - -) to 3.2 (○), 3.0 (●), 2.8 (□), 2.4 (■) or 2.0% (Δ) using skim milk UF permeate. Error bars are excluded to preserve clarity.

The heat stability results agree with the observations reported in Chapter III, where the addition of increasing quantities of SMP to skim milk led to a progressive increase in heat stability, and indicate that the presence of homogenized milk fat did not affect the heat stability trend. The increased heat stability of milk standardized with SMP could be attributed to dilution and a reduced concentration of soluble salts, especially calcium. In principle, dilution should increase the heat stability by reducing the rate of collisions between protein molecules, while salts, especially calcium, are considered to be important in the initial stages of heat-induced casein micelle aggregation (van Boekel *et al.*, 1989a, b).

Sensory quality of protein-standardized milk

Triangle sensory tests showed that it was difficult to distinguish between normal and protein-standardized 2.0% fat milk, whether the milk was 'down'-standardized to 2.6% protein with SMP or 'up'-standardized with α-F or SMR to at least 3.6% protein (Table V-1).

Table V-1. Mean sensory differences between normal 2% fat milk (3.44% protein) and protein-standardized 2% fat milk, determined by triangle tests carried out with 17 individuals. Protein standardization was carried out by enrichment of milk with skim milk ultrafiltration retentate (SMR) or skim milk ultrafiltration permeate (SMP), or the direct of addition of a dried fraction of α-la to 2% fat milk.

Method of protein standardization	*% (w/w) Protein of protein-standardized 2% fat milk*	*Number of correct results*[1]
2% Fat milk + α-fraction	3.6	5 (n.s.)
	3.8	8 (n.s.)
Partly skimmed milk + whole milk + SMR	3.6	5 (n.s.)
	3.8	10 (*)
Partly skimmed milk + whole milk + SMP	3.0	6 (n.s.)
	2.6	7 (n.s.)

[1]: Differences between normal milk and standardized milk were not significant (n.s.) or significant (*) at $\alpha = 0.05$.

At 3.8% protein, the addition of SMR had a marginal effect on flavour; in one of the sensory trials a statistically significant difference occurred ($\alpha = 0.05$), which was just above the threshold of significance. The sensory change was so small that the flavour of the standardized milk was still acceptable. The composition of the α-F indicates that its addition to milk would increase the concentration of total protein, with small changes in the content of lactose and ash. Therefore, the inability of the α-F to modify the flavour of milk implies that the protein α-la is not flavourful, as is the case for most proteins.

As reported in Chapter III, the addition of SMP to milk reduces the concentration of soluble salts, but the similarity in sensory quality between control milk and milk 'down'-standardized with SMP indicate that this change was too small to be detected sensorically, down to at least 2.6% protein.

Conclusions

Altering the protein content of 2.0% fat milk, either 'upward', in the range 3.44 - 4.0% protein, by the direct addition of α-F or by enrichment with SMR, or 'downward', in the range 3.4 - 2.0 % protein, by addition of SMP, caused noticeable changes in heat stability at the unaltered pH (~ 6.7); the presence of SMP caused heat stability to increase whereas with α-F or SMR, heat stability decreased. The heat stability effects were unlikely to be of practical importance, because the heat treatments were well in excess of that required for most industrial operations. The presence of milk fat interfered with heat-induced protein aggregation and/or its observation and made the subjective heat stability assay imprecise. A more objective and sensitive measure of heat stability was achieved by low speed centrifugation of heated milk and quantification of the mass of sediment obtained. Milk standardized up to at least 3.6% protein or down to 2.6% protein, with SMP, had sensory quality equivalent to normal milk.

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- Chapter VI -

Nutritional, sensory and physico-chemical characterization of protein-standardized, ultra-high temperature heated milk*

Introduction

In Chapter III, it was shown that protein 'down' standardization of type A skim milk with skim milk ultrafiltration (UF) permeate or sweet whey UF permeate caused a general increase in heat stability at 140°C, in the pH range 6.4 - 7.1, but the use of acid whey UF permeate had the opposite effect. The presence of homogenized milk fat did not alter the ability of skim milk UF permeate to impart heat stability; in Chapter V it was shown that standardization of 2% fat milk with SMP led to an increase in heat stability, when stability was measured at natural pH. In Chapter IV, it was seen that the use of SMP or SWP for protein standardization 'downward' had little effect on sensory quality, while the use of permeate derived from acid whey had a deleterious effect on sensory quality, especially when permeate was derived from a fermented dairy product.

These studies were carried out on a laboratory scale but were not extended to a more practical situation. Ultra-high temperature (UHT) milk would be an ideal product in which to more fully assess the feasibility of protein standardization procedures. The heat treatments used during the manufacture of UHT milk are relatively severe and thus a practically-applicable assessment of the effect of protein standardization on the heat stability of milk could be obtained. The influence of UF permeate addition on the sensory quality of severely heated milk, such as UHT milk, is unknown. This could differ from its effect on the flavour of pasteurized milk due to the presence of heat-generated, sulphur-containing flavour compounds in UHT milk. Additionally, if a permeate derived from a fermented dairy product was used for standardization, the possibility exists that off-flavours produced during microbial fermentation would be volatilized by heating, especially for direct UHT heating, because of the vacuum flashing of steam, involved in the cooling stage. Furthermore, it is not known how protein standardization affects the age-gelation or sedimentation of UHT milk during storage, the processes which restrict the shelf life of UHT milk to ~ 3 - 6 months.

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The principal factors affecting the manufacture of an acceptable UHT milk are: (1) Thermal stability; milk must tolerate typical heat treatments of 135 - 150°C for 20 - 2 s, achieved by direct or indirect heating (Hinrichs and Kessler, 1995). (2) Physical stability on storage; UHT milk is prone to age-gelation, creaming and sedimentation, which typically restrict its shelf life to 3 - 6 months at ambient temperature (Nieuwenhuijse, 1995). (3) Sensory quality; the flavour of freshly made UHT milk has been described as 'cooked', 'sulphurous' or 'cabbagy', becoming 'stale' or 'oxidized' upon storage (Calvo and Holz, 1992; Andersson and Öste, 1995b; Nursten, 1995). (4) Nutritional quality; heating leads to the destruction of certain vitamins (Andersson and Öste, 1995a), which, though undesirable, may be tolerable in the western diet.

In the present study, milk products with the same target fat content (1.7%), but protein contents in the range 3.36 - 2.6%, were prepared by blending whole milk and skim milk with permeates obtained by UF of skim milk (SMP) or quarg acid whey (FWP), where quarg manufacture involved the fermentation of skim milk. General objectives of this work were to determine the effect of protein standardization on (1) the ability of milk to withstand direct or indirect UHT heating; (2) the nutritional and sensory properties of UHT milk; (3) the storage stability of UHT milk.

Materials and methods

The procedure to prepare protein-standardized, UHT milk was carried out on three separate occasions, using three separately acquired batches of whole milk, skim milk and UF permeate. In general, for each of the three trials, the various analyses were carried out in duplicate and means and standard deviations were calculated using all data (n = 6).

Protein standardization of milk

Skim milk and whole milk were obtained from the Federal Dairy Research Institute, Liebefeld, Switzerland. The UF permeates were kindly provided by Emmi Milch AG, Luzern, Switzerland. The SMP was produced by industrial-scale UF of normal skim milk, using an APV Pasilac DDS plate and frame UF unit equipped with a polysulphone membrane (GR 61 PP) with a cut off of ~20 000 Da. The UF was carried out at 50°C and an average cross membrane pressure of 550 kPa. Industrial-scale UF of quarg acid whey yielded FWP. Quarg manufacture involved fermentation of skim milk at 22°C for 25 h to

pH 4.7, using a commercial culture of lactic acid bacteria (the culture used was not disclosed by the manufacturer). The quarg acid whey produced was ultrafiltered at 47°C and an average cross-membrane pressure of 400 kPa, using a spiral wound, polysulphone membrane, capable of retaining molecules of > 5 nm diameter.

Whole milk, skim milk and UF permeates were kept at 4°C for < 24 h, during which time their fat and protein contents were determined, to enable calculation of the required amounts to be blended to obtain fat- and protein-standardized milk products. About 80 kg of each milk was prepared, containing ~ 1.7% (w/w) fat and ~ 3.4, 3.2, 2.9 or 2.6% (w/w) protein. Control milk contained 3.36% (w/w) protein and was prepared by the sole mixing of whole milk and skim milk. In this study, milk containing SMP is termed S1, S2 or S3 milk, in order of increasing level of addition of permeate (decreasing protein content); likewise, milk with FWP is denoted as F1, F2 or F3 milk.

Ultra-high temperature heating

The UHT milk products were prepared using a pilot scale UHT machine (APV Baker AS, Kolding, Denmark) with direct and indirect heating capabilities and a throughput of ~ 160 L milk h^{-1}. For direct UHT treatment, milk was pre-heated to 89°C, heated directly by steam infusion to 150°C for a 2.7 s holding period, vacuum flash-cooled to 83°C, further cooled to 65°C and homogenized at 18.5 MPa and 65°C under aseptic conditions, using a 3 stage, reciprocating homogenizer. After homogenization, the milk was cooled to 4°C and transferred aseptically into glass bottles. Indirect UHT treatment involved heat treatment of milk by the use of plate heat exchangers throughout. Milk was pre-heated to 65°C, homogenized at 18.5 MPa, with the same homogenizer as used for direct UHT heating, pre-heated to 90°C for 2.4 s, heated to 138°C and held for 1 s, cooled to 4°C, followed by aseptic filling into glass bottles. In some heating trials, the pH of protein-standardized milk was adjusted to 6.8, using 30% NaOH, prior to heating. Milk products were stored at 4 or 25°C, in darkness for up to 12 weeks, the maximum storage time allowed for UHT milk in Switzerland, the country where this work was carried out.

Compositional analyses

Prior to the preparation of protein-standardized milk, the fat and protein content of skim milk, whole milk and the two UF permeate types were determined by standard methods

(IDF, 1987a, 1993). After 1 week of storage at 4°C, the UHT milk products were assayed for total solids (IDF, 1987b), protein (IDF, 1993), fat (IDF, 1987a), lactose (IDF, 1974) and ash (Anonymous, 1987).

The nutritional quality of each protein-standardized, UHT milk was further assessed by measuring the content of certain minerals, after 1 week storage at 4°C. The analysis of minerals was only carried out for direct UHT milk samples, as it was assumed that the type of heat treatment would not affect the absolute concentration of any mineral. Phosphorus and chloride were determined according to IDF (1990, 1988). Sodium, potassium, calcium and magnesium were measured by the following 'in house' procedure: to an exactly know mass (~5 g) of milk, 5 mL of 65% nitric acid was added and the mixture heated at 90°C for 1 h, to completely solubilize all the minerals. The sample was then combusted in an air/acetylene flame where the intensity of light emitted at 589 or 769.9 nm was proportional to the content of sodium or potassium, respectively, while the absorption of light at 422.7 or 285.2 nm was proportional to the content of calcium or magnesium, respectively. Spectral measurements were carried out using a Video 22E atomic absorption/atomic emission spectrophotometer (Instrumentation Laboratory Inc., Lexington, MA, USA).

The concentrations of certain vitamins were determined to further characterize the nutritional value of the protein-standardized, UHT milk products. Analyses were carried out for both direct and indirect UHT milk, as it is known that certain vitamins can be destroyed by heating. The concentrations of vitamin B_1 and B_2 were determined, after 1 week storage at 4°C, according to Tagliaferri *et al.* (1992a, b). After 1 week storage at 4°C, the concentrations of vitamin A and E were determined together by a new method (Anonymous, 1995) developed at the Swiss Federal Dairy Research Institute.

The control milk or protein-standardized milk products were assayed for non-casein nitrogen (IDF, 1964) and non-protein nitrogen, before, as well as 1, 4, or 12 weeks after, UHT heating. The non-protein nitrogen was determined by addition of 12% trichloroacetic acid to milk to precipitate the proteins, filtration of the milk and measurement of the total nitrogen in the filtrate. Titratable acidity was measured according to the method of Anonymous (1993), before and 1, 4 or 12 weeks after heating, as a further indicator of possible changes occurring upon storage. Results for were expressed as °Th (mL of 0.1 N NaOH needed to titrate 100 mL of milk).

Apparent viscosity

The physical stability of UHT milk products was assessed by measuring their apparent viscosities after 1, 4 or 12 weeks of storage at 4 or 25°C. The apparent viscosity (η_{app}) was determined, at 25°C, according to the following equation:

$$\eta_{app} = \frac{t}{t_0} \cdot \eta_0 \cdot \frac{d_{milk}}{d_o}$$

where t = time for milk to flow through the bulb of a capillary viscometer; t_0 = time for water to flow through the bulb of a capillary viscometer; η_0 = viscosity of water (0.8904 mPa.s at 25°C); d_0 = density of water; and d_{milk} = density of milk. The capillary viscometer, manufactured by Jena Glaswerk (Schott & Gen., Mainz, Germany), was submerged in a 25°C water bath and was used to compare the flow rates of water and milk samples. The density of UHT milk samples was measured, at 25°C, using an Auto Paar, DMA 55 (Auto Paar K.G., Graz, Austria) densitometer.

Sedimentation

As a further indicator of the physical stability of UHT milk samples, sedimentation tendencies were determined. Sedimentation was calculated as g wet sediment per kg milk, after determining the mass of sediment remaining upon inversion and drainage, for 30 min, of an exactly known mass of milk from a 1 L capacity bottle. In some UHT milk products, protein aggregation was so extensive that phase separation occurred and the sediment had a custard-like consistency. In these samples, the mass of sediment was estimated after careful decantation of the supernatant. The composition (total solids, protein, lactose, fat, ash, calcium and phosphorus) of the sediment obtained from some milk samples was determined, by the same methods as above.

Sensory evaluation

A panel consisting of 6 trained and certified individuals, all from the Federal Dairy Research Institute, Liebefeld, Switzerland was used for sensory evaluation. The direct or indirect UHT milk products were evaluated on separate days with no more than 7 samples per panelist on each occasion. Milk samples were equilibrated to ~ 15°C and aliquots (~ 50 mL) placed in small plastic cups. A 10 point hedonic sensory score system was

used, in which odour was assigned a maximum (most liked) weighting of 2 (5 x 0.4) points and taste a maximum of 8 (5 x 1.6) points. The panelists were encouraged to give descriptive comments, assisted by a list of possible defects.

Results and discussion

Heat stability

In preliminary experiments, each milk product was UHT heat-treated at its native pH, corresponding to pH 6.7 for control milk or milk with SMP. In the case of these milk products, immediately after direct or indirect UHT heating, there were no visible signs of destabilization. The result was expected; normal milk can be heated for up to 20 min at 140°C without visible protein coagulation and its heat stability is even greater upon the addition of SMP (see Chapters III and V). In accordance with normal industrial practice, control milk or milk with SMP were UHT-heated at their natural pH (~ 6.7).

Addition of increasing amounts of FWP to raw milk caused the pH to decline from ~ 6.7 to values in the range ~ 6.50 - 6.65. This minor pH shift was accompanied by a large decline in heat stability; direct UHT heating of milk standardized to 3.14 % (w/w) protein, corresponding to the lowest level of added FWP, led to coagulation which could be observed in the steam infusion chamber. To evade the possibility of damage to the UHT machine, other milk products standardized with FWP were not tested; destabilization would probably have been even greater, due to the higher levels of FWP.

As seen in Chapter III, the addition of acid whey UF permeate to skim milk caused a general decline in heat stability over the initial pH range 6.4 - 7.1 (Figure III-4); this was especially evident at the unaltered pH of milk (Figure III-5), where some samples heat-coagulated at 90°C. Reduced heat stability was attributed to the high calcium content of the permeate, which shifted the heat stability-pH curve of type A milk to more alkaline pH values and led to a faster rate of heat-induced pH decline. An exception was an increased heat stability of milk with acid whey UF permeate at initial pH 6.8; extra calcium may have inhibited the formation of soluble complexes between β-lg and κ-casein and hence heat labile, κ-casein-depleted micelles. In view of this observation, further UHT heating trials were carried out after adjusting milk with FWP to initial pH 6.8. It was found that during direct or indirect UHT heating, visible coagulation of milk did not occur; henceforth, all milk products with FWP were neutralized to pH 6.8 before UHT heating.

The pH of all milk products with FWP declined from the neutralized values (6.8) to ~ 6.7, upon direct or indirect UHT heating, but the pH of milk containing SMP was unchanged (~ 6.7). In Chapter III it was seen that skim milk with acid whey UF permeate experienced a rapid pH decline when heated at 140°C, possibly due to its relatively high calcium content which may have increased the precipitation of calcium phosphate salts, with release of H^+ ions.

Composition

Compositional data confirmed the expected differences between the two permeate types especially with respect to lactose and mineral content (Tables VI-1 and VI-2). Much of the minerals in milk are associated with casein and other proteins, and thus would be retained during UF (Green *et al.*, 1984; Premaratne and Cousin, 1991), accounting for the relatively low mineral content of the SMP. There also exists the possibility that certain minerals were not completely permeable through UF membranes or had an increased retention due to the 'Donnan potential' (see section 2.3.3 of Chapter II). The concentrations of individual minerals, except phosphorus, in the FWP were very similar to that in normal milk, indicating that acidification caused dissolution of salts from casein micelles, allowing the entire mineral component to permeate the UF membrane. Therefore in milk with FWP, the concentration of total minerals was similar to that in normal milk, but the concentration of soluble minerals was greater; this appeared to have a major influence on its physical stability after UHT heating (see below). Control milk had ~ 22 mg 100 g^{-1} more phosphorus than the FWP, similar to a value reported by Walstra and Jenness (1984) for organic phosphorus in milk (21.6 mg 100 g^{-1}). Organic phosphorus is esterified to casein and therefore would be expected not to undergo acid dissociation.

The lower lactose content of the FWP, as compared to SMP, can be explained by the fact that the FWP originated from quarg; the manufacture of quarg involves fermentation of skim milk, whereby some of the lactose is converted into lactic acid. The 'protein' in the UF permeates was probably non-protein nitrogen; low molecular weight peptides, amino acids, urea and other nitrogenous compounds capable of permeating through UF membranes.

Table VI-1. Mean (n = 6) composition, measured after 1 week of storage at 4°C, of skim milk UF permeate (SMP), quarg acid whey permeate (FWP) and direct UHT milk products, whereby S1, S2 or S3 denote the presence of increasing amounts of SMP and F1, F2 or F3 indicate the presence of increasing amounts of FWP. Bracketed numbers indicate the standard deviation of the data.

Product	*Total solids*	*Protein*	*Fat*	*Lactose*	*Ash*
	% (w/w)				
SMP	5.38 (0.18)	0.19 (0.04)	0.0 (0.0)	4.97 (0.17)	0.46 (0.01)
FWP	5.77 (0.14)	0.22 (0.06)	0.0 (0.0)	4.32 (0.06)	0.73 (0.01)
Control milk	10.75 (0.37)	3.36 (0.03)	1.69 (0.08)	4.98 (0.24)	0.74 (0.01)
S1 milk	10.54 (0.17)	3.18 (0.05)	1.71 (0.13)	4.99 (0.25)	0.73 (0.02)
S2 milk	10.07 (0.24)	2.90 (0.06)	1.63 (0.09)	5.02 (0.23)	0.71 (0.02)
S3 milk	9.58 (0.22)	2.55 (0.05)	1.57 (0.23)	4.99 (0.22)	0.68 (0.02)
F1 milk	10.42 (0.40)	3.14 (0.09)	1.7 (0.20)	4.96 (0.28)	0.72 (0.04)
F2 milk	10.08 (0.36)	2.91 (0.03)	1.63 (0.12)	4.86 (0.29)	0.75 (0.02)
F3 milk	9.68 (0.16)	2.58 (0.05)	1.58 (0.25)	4.78 (0.33)	0.79 (0.03)

Table VI-2. Mean (n = 6) concentration of specific minerals, measured after 1 week of storage at 4°C, of skim milk UF permeate (SMP), quarg acid whey permeate (FWP) and direct UHT milk products, whereby S1, S2 or S3 denote the presence of increasing amounts of SMP and F1, F2 or F3 indicate the presence of increasing amounts of FWP. Bracketed numbers indicate the standard deviation of the data.

Product	*Calcium*	*Magnesium*	*Phosphorus*	*Potassium*	*Sodium*	*Chloride*
	mg 100 g^{-1} milk					
SMP	27.9 (0.2)	0.9 (0.1)	6.8 (0.8)	24.0 (1.4)	ND[1]	ND[1]
FWP	122.4 (8.3)	8.7 (0.2)	67.0 (1.3)	161.0 (6.0)	37.8 (4.0)	94.0 (2.7)
Control milk	118.5 (5.4)	9.8 (0.3)	89.0 (4.2)	168.0 (9.9)	39.8 (0.1)	101.5 (3.5)
S1 milk	110.5 (0.7)	9.7 (0.3)	86.0 (1.4)	165.0 (11.3)	39.0 (4.5)	101.5 (2.1)
S2 milk	106.5 (4.2)	9.2 (0.1)	81.0 (1.4)	164.5 (10.6)	38.0 (6.9)	100.5 (2.1)
S3 milk	95.5 (5.3)	8.5 (0.2)	73.5 (0.7)	150.5 (4.9)	38.0 (3.9)	102.0 (2.8)
F1 milk	117.5 (8.1)	9.2 (0.4)	86.0 (0.1)	153.5 (0.7)	39.8 (4.5)	99.5 (3.5)
F2 milk	118.0 (8.6)	9.1 (0.7)	84.0 (4.2)	154.5 (6.3)	52.4 (0.5)	99.0 (2.8)
F3 milk	121.0 (9.9)	9.3 (0.5)	82.5 (0.7)	157.5 (4.9)	65.9 (3.8)	98.5 (0.7)

[1]: Not determined

Analyses of the UHT milk products gave ~ 3.4, 3.2, 2.9 or 2.6% (w/w) protein and ~ 1.7% (w/w) fat, indicating that the raw materials had been combined appropriately. Control milk and milk with SMP had similar concentrations of lactose, whereas milk with FWP had a reduced level. Standardization of milk with SMP caused a slight reduction in the total ash content, more specifically in calcium, magnesium, phosphorus and potassium, while the concentration of sodium and chloride were unchanged. Standardization of milk with FWP had very little effect on the content of total ash or individual minerals such as calcium, phosphorus, magnesium or chloride. The mineral composition of bovine milk exhibits a significant degree of natural variability; calcium ranges from ~ 110 - 130 mg L^{-1}; potassium from ~ 110 - 170 mg L^{-1} (Flynn and Power, 1985). This natural variability should be considered when evaluating the importance of the changes in mineral composition caused by protein standardization.

Enrichment of milk with SMP or FWP caused the concentrations of vitamin A (retinol) and vitamin E (mostly α-tocopherol) to fluctuate slightly, but without a clear trend (Table VI-3). Retinol and α-tocopherol are fat-soluble vitamins which accounts for their absence in the fat-free UF permeates. Likewise, the concentration of these vitamins in skim milk should have been very low. Because control and protein-standardized milk products had approximately the same fat content, the concentration of fat soluble vitamins was expected to be unchanged, as indeed seemed to be the case; the minor variations in vitamin concentration were probably caused by fluctuations in the concentration of fat (Table VI-1). The type of UHT heating had no clear effect on the concentration of retinol or α-tocopherol in milk, indicating a high heat stability of these vitamins, as earlier documented (Ford *et al.*, 1969; Burton *et al.*, 1970; Le Maguer and Jackson, 1983).

Standardization of milk with either SMP or FWP did not alter the concentration of vitamin B_1 (thiamine). Most (83 - 95%) of the thiamine in milk is not bound to proteins and should be freely permeable through UF membranes (Premaratne and Cousin, 1991) and thus the concentration of thiamine in the permeates should be similar to that in normal milk and hence protein-standardized milk. In agreement with Lembke *et al.* (1968), levels of thiamine in direct or indirect UHT milk samples were almost identical, indicating that the greater heat load applied to indirect UHT milk did not lead to extra destruction of the vitamin. At the same time, however, the concentrations of thiamine in the UHT milk samples were about half that of the reported (Fink and Kessler, 1985) value for raw milk

(35 - 45 mg L^{-1}), concurring with the observed heat sensitivity of this vitamin (Ford *et al.*, 1969; Burton *et al.*, 1970). Considering that information on the kinetics of the thermal destruction of thiamine in milk is rather equivocal (Andersson and Öste, 1995a), the similar values for thiamine concentration in the direct and indirect UHT milk products cannot be readily explained.

Table VI-3. Concentrations, measured after 1 week of storage at 4°C, of vitamins A, E, B_1 and B_2 in skim milk UF permeate (SMP), quarg acid whey UF permeate (FWP) and direct or indirect UHT milk products, where the terms S1, S2 or S3 denote the presence of increasing amounts of SMP, while F1, F2 or F3 indicate increasing amounts of FWP.

Product	*Vitamin A (retinol)*		*Vitamin E (α-tocopherol)*		*Vitamin B_1 (thiamine)*		*Vitamin B_2 (riboflavin)*	
	Concentration (mg 100 g^{-1})							
SMP	Not detected[1]		Not detected[1]		13		128	
FWP	Not detected[1]		Not detected[1]		16		135	
	Direct	Indirect	Direct	Indirect	Direct	Indirect	Direct	Indirect
Control milk	31	22	81	59	21	20	148	153
S1 milk	23	18	62	55	21	20	156	139
S2 milk	21	19	56	52	21	21	156	148
S3 milk	16	19	39	50	20	22	143	146
F1 milk	27	20	71	58	20	21	144	153
F2 milk	23	21	60	58	20	20	152	150
F3 milk	17	21	45	55	19	20	149	140

[1]: The concentration of fat soluble vitamins in the UF permeates were below the limit of determination for the analytical equipment used.

Premaratne and Cousin (1991) observed that UF of milk by a five fold concentration factor led to about a 1.5 fold increase (from 178 to 251 µg 100 g^{-1} milk) in the concentration of vitamin B_2 (riboflavin), which was attributed to its reported (Hartman and Dryden, 1965) association with the casein proteins and the co-enzymes flavin adenine dinucleotide and flavin mononucleotide. This observation is consistent with the present results, which show that the concentrations of riboflavin in the SMP or FWP were lower than in normal skim milk. Thus, addition of either permeate to milk should have reduced slightly the concentration of riboflavin. However, no consistent effect of standardization on the level of riboflavin occurred, possibly because the levels of permeate used were

relatively low. Furthermore, the concentration of riboflavin in the skim or whole milk used to prepare the control or protein-standardized milk products was unknown which may have contributed to the fluctuations in the riboflavin content of the UHT milk products. The concentration of riboflavin appeared not to be influenced by the type of UHT treatment used, which was expected in view of its reported high heat stability (Oamen *et al.*, 1989).

Non-casein nitrogen

Non-casein nitrogen (NCN) refers to nitrogenous compounds in milk which are soluble at pH 4.6; these include the whey proteins, proteose peptones, oligopeptides and free amino acids. Direct or especially indirect UHT heating caused the concentration of NCN to decline (Table VI-4), indicating that heat denaturation of serum proteins occurred, resulting in their insolubilization when the pH was reduced to 4.6. Whey protein denaturation would take place to a greater extent in the more severely heated indirect UHT milk products resulting in a lower concentration of NCN. The loss of solubility of whey proteins caused by heating is the basis of several methods used to classify milk, evaporated milk and milk powders (Pellegrino *et al.*, 1995).

Protein standardization caused a small reduction in the NCN content of milk, evident before or after UHT heating and throughout storage and clearly related to the low concentration of NCN in the permeates, especially in the SMP. The low NCN content in the UF permeates was expected, because of the impermeability of UF membranes to whey proteins. The greater content of NCN in the FWP was probably due to the catabolism of protein during the fermentation of milk, involved in quarg manufacture; utilization of casein by lactic acid bacteria requires its hydrolysis into small peptides and free amino acids, capable of being transported across the cell membrane (Pritchard and Coolbear, 1993).

Storage of milk at 4 or 25°C caused the NCN content of all milk samples to increase slightly. This indicates that hydrolysis of protein occurred during storage to liberate polypeptides, small peptides and free amino acids, soluble at pH 4.6. In unconcentrated UHT milk, hydrolysis of casein is probably caused by plasmin and possibly bacterial proteinases (Kokak and Zadow, 1985; Manji *et al.*, 1986; Alkanhal *et al.*, 1994; Harwalkar 1992; Nieuwenhuijse, 1995). The products of hydrolysis include various kinds of oligopeptides, which are soluble at pH 4.6 and thus would contribute to an increased concentration of NCN. The increase in NCN was slightly greater at 25 than at 4°C,

Table VI-4. Influence of protein standardization, type of UHT heat treatment and temperature of storage on the mean non-casein nitrogen content of milk. The terms S1, S2 or S3 indicate milk standardized with increasing amounts of skim milk UF permeate; similarly, F1, F2 or F3 denote milk with increasing quantities of fermented acid whey UF permeate. Bracketed numbers are the standard deviation of each data point.

Heat treatment; storage temperature	*Control milk*	*S1 milk*	*S2 milk*	*S3 milk*	*F1 milk*	*F2 milk*	*F3 milk*
	Non-casein nitrogen (mmol N kg^{-1})						
Before heating	85.0 (2.8)	79.5 (2.1)	74.0 (2.8)	69.5 (3.5)	84.5 (3.5)	79.5 (3.5)	74.5 (7.2)
Direct UHT; 4°C							
1 week	43.7 (0.6)	40.3 (1.2)	38.7 (0.6)	36.7 (1.5)	41.0 (1.7)	42.0 (1.4)	41.5 (3.5)
4 weeks	42.7 (2.3)	43.7 (2.5)	40.7 (0.6)	40.0 (2.8)	42.3 (1.2)	44.5 (0.7)	43.5 (0.7)
Direct UHT; 25°C							
4 weeks	45.0 (2.2)	52.5 (0.7)	47.0 (5.7)	40.5 (0.7)	46.3 (1.5)	46.5 (3.5)	45.5 (0.7)
12 weeks	52.0 (3.0)	53.0 (3.0)	52.0 (2.1)	52.5 (2.1)	53.0 (2.8)	51.3 (2.3)	52.5 (6.4)
Indirect UHT; 4°C							
1 week	37.0 (2.8)	36.5 (3.5)	35.5 (2.1)	33.0 (1.4)	36.0 (1.4)	36.5 (2.1)	37.5 (2.1)
4 weeks	38.7 (2.3)	38.3 (1.5)	37.7 (2.1)	34.5 (2.1)	38.7 (2.1)	41.3 (6.1)	37.5 (0.7)
12 weeks	44.7 (1.5)	44.3 (1.2)	42.3 (0.6)	40.0 (1.4)	45.5 (0.7)	44.0 (2.0)	45.0 (2.1)
Indirect UHT; 25°C							
4 weeks	43.5 (0.7)	42.7 (4.7)	39.7 (2.5)	35.5 (0.7)	42.0 (1.4)	38.5 (2.1)	41.5 (3.5)
12 weeks	48.5 (0.7)	50.0 (1.4)	49.7 (3.5)	41.5 (0.7)	45.5 (2.1)	43.5 (3.5)	42.0 (7.0)

probably because of greater residual enzymatic activity at the higher temperature. Additionally, plasminogen, the inactive precursor of plasmin, is converted more rapidly to plasmin, at a higher storage temperature (Manji *et al.*, 1986). Protein hydrolysis occurred at a slightly slower rate in the indirect UHT milk products, possibly because the more severe heat treatment led to a greater inactivation of plasmin.

Non-protein nitrogen

The non-protein nitrogen (NPN) indicates nitrogenous compounds soluble in 12% trichloroacetic acid; this includes low molecular weight oligopeptides and free amino acids, but excludes whey proteins. After direct or especially indirect UHT heating, the NPN content of milk increased slightly (Table VI-5), indicating the generation of peptides or possibly free amino acids during UHT heating. Standardization with SMP had no effect on the NPN content of milk, but when FWP was used the concentration of NPN increased slightly, evident before or after UHT heating; this was due to the similar NPN content of SMP (18 mmol kg^{-1}) or control milk (21 mmol kg^{-1}), while FWP had a higher NPN content (32 mmol kg^{-1}). The similarity in the NPN content of normal milk and SMP indicates that most low molecular weight nitrogenous compounds present in the milk permeated the UF membranes. The higher NPN content of the FWP implies the occurrence of protein hydrolysis, caused by the activity of bacteria during quarg manufacture; as described above this may have also contributed to a higher NCN content.

Storage, at 4 or 25°C, had a negligible effect on the concentration of NPN in any UHT milk sample. This suggests that the main effect of protein hydrolysis was to increase the content of relatively large polypeptides (NCN) and that smaller-sized peptides and free amino acids (NPN) were not liberated, even after 12 weeks storage at 25°C. Thus, the concentration of NCN is a more sensitive index of proteolysis, at least for relatively short storage periods of UHT milk, in agreement with the discussion of Nieuwenhuijse (1995).

pH

The effects of protein standardization, direct or indirect UHT heating and subsequent storage on the pH of milk are shown in Table VI-6. The changes in the pH were small, but the following trends are noted: (1) The pH of milk containing AWP declined from ~ 6.8 (adjusted pH) to ~ 6.7, after direct or indirect UHT heating. (2) Upon storage, the pH of

Table VI-5. Influence of protein standardization, type of UHT heat treatment and temperature of storage on the mean non-protein nitrogen content of milk. The terms S1, S2 or S3 indicate milk standardized with increasing amounts of skim milk UF permeate; similarly, F1, F2 or F3 denote milk with increasing quantities of fermented acid whey UF permeate. Bracketed numbers are the standard deviation of each data point.

Heat treatment; storage temperature	*Control milk*	*S1 milk*	*S2 milk*	*S3 milk*	*F1 milk*	*F2 milk*	*F3 milk*
	Non-protein nitrogen (mmol N kg^{-1})						
Before heating	20.0 (1.4)	20.0 (1.4)	20.0 (1.4)	20.0 (1.4)	21.0 (1.4)	21.5 (0.7)	22.0 (1.4)
Direct UHT; 4°C							
1 week	21.7 (1.5)	21.7 (1.5)	21.0 (1.0)	21.0 (1.7)	22.0 (1.7)	23.5 (0.7)	24.5 (0.7)
4 weeks	22.7 (0.6)	22.7 (0.6)	21.7 (0.6)	23.0 (2.0)	22.7 (0.6)	24.5 (0.7)	25.5 (0.7)
12 weeks	21.7 (1.5)	21.3 (1.2)	22.3 (0.6)	20.7 (1.5)	21.7 (0.6)	23.0 (0.0)	24.0 (0.0)
Direct UHT; 25°C							
4 weeks	25.0 (5.7)	26.7 (7.2)	24.3 (3.2)	24.3 (4.2)	22.3 (1.2)	23.5 (0.7)	23.5 (0.7)
12 weeks	22.0 (0.6)	25.5 (2.1)	24.0 (1.4)	24.0 (1.4)	23.5 (0.7)	23.7 (1.2)	25.0 (0.1)
Indirect UHT; 4°C							
1 week	22.0 (0.0)	22.0 (0.0)	21.5 (0.7)	21.5 (0.7)	22.5 (0.7)	23.0 (1.4)	24.5 (0.7)
4 weeks	22.3 (0.6)	22.0 (0.0)	21.7 (0.6)	21.5 (0.7)	22.4 (1.4)	23.0 (0.1)	24.5 (0.7)
12 weeks	21.7 (0.6)	21.0 (1.0)	21.3 (0.6)	21.0 (1.0)	22.5 (0.7)	23.0 (2.0)	26.0 (1.4)
Indirect UHT; 25°C							
4 weeks	22.0 (1.4)	21.3 (1.2)	21.3 (0.6)	21.5 (0.7)	26.5 (4.9)	22.5 (0.7)	24.0 (1.4)
12 weeks	23.0 (1.0)	23.0 (1.2)	22.0 (1.0)	22.5 (0.7)	23.5 (0.7)	24.0 (0.0)	25.0 90.7)

Table VI-6. Influence of protein standardization, type of UHT heat treatment and temperature of storage on the mean pH of milk. The terms S1, S2 or S3 indicate milk standardized with increasing amounts of skim milk UF permeate; similarly, F1, F2 or F3 denote milk with increasing quantities of fermented acid whey UF permeate. Bracketed numbers are the standard deviation of each data point.

Heat treatment; storage temperature	*Control milk*	*S1 milk*	*S2 milk*	*S3 milk*	*F1 milk*	*F2 milk*	*F3 milk*
	pH						
Before heating	6.67 (0.02)	6.70 (0.02)	6.69 (0.03)	6.67 (0.02)	6.81 (0.02)	6.80 (0.02)	6.80 (0.03)
Direct UHT; 4°C							
1 week	6.72 (0.02)	6.73 (0.03)	6.72 (0.03)	6.72 (0.02)	6.72 (0.02)	6.70 (0.04)	6.68 (0.04)
4 weeks	6.69 (0.00)	6.70 (0.01)	6.70 (0.00)	6.71 (0.01)	6.69 (0.01)	6.68 (0.02)	6.69 (0.02)
12 weeks	6.67 (0.03)	6.66 (0.02)	6.66 (0.01)	6.69 (0.02)	6.64 (0.06)	6.66 (0.01)	6.66 (0.02)
Direct UHT; 25°C							
4 weeks	6.53 (0.04)	6.66 (0.02)	6.65 (0.02)	6.63 (0.04)	6.66 (0.03)	6.66 (0.02)	6.64 (0.03)
12 weeks	6.56 (0.03)	6.54 (0.03)	6.50 (0.05)	6.56 (0.04)	6.49 (0.06)	6.49 (0.06)	6.46 (0.03)
Indirect UHT; 4°C							
1 week	6.67 (0.02)	6.69 (0.01)	6.67 (0.01)	6.65 (0.02)	6.69 (0.01)	6.63 (0.02)	6.63 (0.02)
4 weeks	6.62 (0.02)	6.59 (0.03)	6.61 (0.03)	6.62 (0.02)	6.59 (0.03)	6.59 (0.01)	6.59 (0.02)
12 weeks	6.58 (0.04)	6.59 (0.03)	6.59 (0.02)	6.61 (0.03)	6.61 (0.03)	6.59 (0.04)	6.59 (0.04)
Indirect UHT; 25°C							
4 weeks	6.59 (0.06)	6.60 (0.03)	6.53 (0.06)	6.59 (0.06)	6.58 (0.05)	6.46 (0.04)	6.37 (0.07)
12 weeks	6.50 (0.04)	6.49 (0.10)	6.47 (0.06)	6.44 (0.01)	6.50 (0.08)	6.46 (0.01)	6.39 (0.04)

all milk samples declined, especially for indirect UHT milk at 25°C. (3) Protein standardization did not appear to interact with the effect of storage on pH, except for F3 milk samples, which displayed lower pH values, upon storage at 25°C.

A decline in the pH of UHT milk upon storage has been reported previously (Kokak and Zadow, 1985; Manji *et al.*, 1986; Kondal Reddy *et al.*, 1991; Alkanhal *et al.*, 1994). Andrews *et al.* (1977) postulated that upon storage of UHT milk, a slow Maillard reaction took place between the ε-amino groups of proteins and hydroxyl groups of lactose, such that H^+ ions were released and the rate of this reaction would increase with increasing temperature. In the next Chapter, it is shown that the Maillard reaction can take place during storage of UHT milk, notably at a much faster rate at 25°C than at 4°C. This was revealed by an increased concentration of lactuloselysine, a product of the early Maillard reaction, during storage.

Within the limits of this study, no solid explanation can be offered for the greater decline in pH of the F3 milk, upon storage. It is possible that the higher content of soluble salts in this milk played a role and that upon storage shifts in salt equilibria occurred to release H^+ ions.

Titratable acidity

The titratable acidity (TA) of milk did not change much upon storage at 4 or 25 °C (Table VI-7). The TA of milk should exhibit a similar variation to pH, as both are related to the activity of H^+ ions (aH^+) in milk. However, due to the logarithmic nature of the pH scale, in the pH range 6 - 7, large changes in pH would be accompanied by very small changes in the aH^+; if the pH of milk decreased from 6.7 to 6.3, then, by calculation, aH^+ would increase by 0.301 mM, contributing to an increase in TA of 0.000301 °Th. Therefore, the changes in pH which occurred were probably accompanied by finite, but very small changes in TA, which could not be detected. Walstra and Jenness (1984) pointed out that the TA of milk is most sensitive to changes in the buffering constituents of milk, such as proteins, soluble and colloidal salts and organic acids, rather than the aH^+. The fact that TA did not change during storage indicates that the buffering properties of milk were unaltered, despite the fact that protein in milk samples with FWP became extremely unstable upon storage (see below).

Table VI-7. Influence of protein standardization, type of UHT heat treatment and temperature of storage on the mean titratable acidity of milk. The terms S1, S2 or S3 indicate milk standardized with increasing amounts of skim milk UF permeate; similarly, F1, F2 or F3 denote milk with increasing quantities of fermented acid whey UF permeate. Bracketed numbers are the standard deviation of each data point.

Heat treatment; storage temperature	*Control milk*	*S1 milk*	*S2 milk*	*S3 milk*	*F1 milk*	*F2 milk*	*F3 milk*
	Titratable acidity (°Th; mL 0.1 N NaOH 100 mL^{-1} milk)						
Direct UHT; 4°C							
1 week	14.0 (0.2)	13.6 (0.2)	13.2 (0.2)	12.5 (0.4)	14.1 (0.3)	14.2 (0.4)	14.4 (0.5)
4 weeks	13.9 (0.1)	13.7 (0.1)	12.9 (0.1)	12.6 (0.6)	13.8 (0.0)	13.8 (0.0)	13.7 (0.2)
12 weeks	13.9 (0.2)	13.7 (0.7)	13.2 (0.8)	12.2 (0.3)	13.7 (0.4)	13.0 (0.2)	13.2 (0.4)
Direct UHT; 25°C							
4 weeks	13.8 (0.5)	14.1 (0.7)	13.0 (0.1)	12.7 (0.5)	13.7 (0.2)	14.1 (0.2)	14.1 (0.3)
12 weeks	14.4 (0.4)	15.2 (0.5)	13.7 (0.2)	13.1 (0.3)	14.4 (0.1)	14.8 (0.1)	13.7 (1.4)
Indirect UHT; 4°C							
1 week	16.0 (0.5)	15.5 (0.3)	14.9 (0.2)	14.0 (0.5)	15.5 (0.3)	15.9 (0.1)	16.2 (0.2)
4 weeks	15.8 (0.3)	15.2 (0.5)	14.8 (0.6)	13.5 (0.0)	15.3 (0.0)	15.5 (0.0)	15.7 (0.1)
12 weeks	16.3 (0.7)	15.5 (0.5)	14.9 (0.5)	13.7 (0.1)	15.6 (0.1)	16.5 (0.7)	16.4 (0.6)
Indirect UHT; 25°C							
4 weeks	15.8 (1.1)	15.2 (0.6)	14.7 (0.9)	14.3 (1.3)	14.4 (1.1)	15.5 (0.1)	16.2 (0.3)
12 weeks	16.1 (0.3)	15.8 (1.2)	15.1 (0.3)	14.2 (0.3)	16.1 (0.2)	16.2 (0.0)	16.5 (0.6)

Apparent viscosity

The apparent viscosities of UHT milk products showed two distinct trends (Table VI-8): (1) Standardization of milk with increasing amounts of SMP caused a gradual decline in viscosity. (2) The F1 milk had a lower viscosity than control milk, but F2 or especially F3 milk products developed very high viscosities during storage.

Due to the absence of protein and fat, the viscosities of the UF permeates were low (1.08 mPa.s for SMP; 1.13 mPa.s for FWP) and their addition to milk was expected to cause the viscosity to decrease, as was observed for S1, S2, S3 or F1 milk, which had consistently lower viscosities than control milk. Age-gelation occurs normally after 6 - 24 months storage, whereupon the apparent viscosity increases suddenly to > 10 mPa.s (Kokak and Zadow, 1985; Kondal Reddy *et al.*, 1991); by this definition, none of the milk products with SMP (S1, S2 or S3 milk) or with the lowest level of FWP (F1 milk) underwent age-gelation. The F2 or F3 milk products exhibited very high viscosities, indicating a destabilization effect caused by the presence of FWP, and consistent with the presence of very large amounts of sediment in these milk samples, as discussed below.

Sedimentation

Sedimentation tendencies are shown in Table VI-9. Although the matrix of data is rather complex, the following trends can be seen: (1) Standardization of milk with SMP caused small, but consistent reductions in sedimentation, evident for both direct or indirect UHT milk products. (2) The amount of sediment obtained from control milk or milk with SMP increased gradually during storage, the temperature of storage having little effect on the trend. (3) In general, milk with FWP displayed very high sedimentation upon storage.

The sedimentation of UHT milk would be affected by viscosity and the number, size and density of the sedimenting particles. Therefore, factors leading to increased intermolecular protein interactions, including heat treatment and an increased concentration of protein and salt should increase the rate of sedimentation. Dalgleish (1992) argued that sedimentation in UHT milk was due to the action of gravity on casein micelles; by calculation it was shown that native micelles could sediment upon prolonged storage of UHT milk and limited aggregation in casein micelles caused a large increase in the rate of sedimentation. For milk with SMP, the reduced concentration of casein micelles, may explain the reduced rate of sedimentation observed; presumably, the lower concentration of

Table VI-8. Influence of protein standardization, type of UHT heat treatment and temperature of storage on the mean apparent viscosity of milk. The terms S1, S2 or S3 indicate milk standardized with increasing amounts of skim milk UF permeate; similarly, F1, F2 or F3 denote milk with increasing quantities of fermented acid whey UF permeate. Bracketed numbers are the standard deviation of each data point.

Heat treatment; storage temperature	*Control milk*	*S1 milk*	*S2 milk*	*S3 milk*	*F1 milk*	*F2 milk*	*F3 milk*
	Apparent viscosity (mPa.s)						
Direct UHT; 4°C							
1 week	1.74 (0.03)	1.72 (0.02)	1.64 (0.03)	1.54 (0.03)	1.65 (0.05)	1.70 (0.21)	1.62 (0.09)
4 weeks	1.93 (0.24)	1.73 (0.02)	1.63 (0.02)	1.55 (0.04)	1.67 (0.11)	1.80 (0.20)	ND[1]
12 weeks	1.84 (0.02)	1.77 (0.02)	1.69 (0.02)	1.58 (0.03)	1.50 (0.03)	1.80 (0.40)	ND[2]
Direct UHT; 25°C							
4 weeks	1.64 (0.04)	1.58 (0.04)	1.54 (0.05)	1.47 (0.01)	1.84 (0.70)	1.60 (0.10)	ND[1]
12 weeks	1.73 (0.06)	1.62 (0.07)	1.53 (0.01)	1.50 (0.09)	1.61 (0.00)	1.72 (0.21)	ND[2]
Indirect UHT; 4°C							
1 week	1.69 (0.01)	1.63 (0.01)	1.59 (0.04)	1.73 (0.15)	1.62 (0.07)	1.81 (0.06)	ND[1]
4 weeks	1.68 (0.01)	1.67 (0.01)	1.58 (0.01)	1.54 (0.08)	1.65 (0.02)	2.21 (0.37)	ND[2]
12 weeks	1.73 (0.05)	1.66 90.04)	1.59 (0.04)	1.63 (0.03)	1.68 90.01)	1.83 (0.12)	ND[2]
Indirect UHT; 25°C							
4 weeks	1.55 (0.01)	1.63 (0.07)	1.51 (0.01)	1.46 (0.01)	1.52 (0.03)	1.58 (0.10)	ND[2]
12 weeks	1.53 (0.02)	1.53 (0.01)	1.49 (0.02)	1.40 (0.01)	1.51 (0.03)	1.63 (0.17)	ND[2]

[1]: The viscosity of these samples was not determined, because excessive sedimentation prevented the flow of sample through the capillary viscometer.

Table VI-9. Influence of protein standardization, type of UHT heat treatment and temperature of storage on the mean mass of sediment obtained from 1 kg of milk. The terms S1, S2 or S3 indicate milk standardized with increasing amounts of skim milk UF permeate; similarly, F1, F2 or F3 denote milk with increasing quantities of fermented acid whey UF permeate. Bracketed numbers are the standard deviation of each data point.

Heat treatment; storage temperature	*Control milk*	*S1 milk*	*S2 milk*	*S3 milk*	*F1 milk*	*F2 milk*	*F3 milk*
	g sediment kg^{-1} milk						
Direct UHT; 4°C							
1 week	2.1 (0.6)	1.5 (0.2)	1.6 (0.2)	1.5 (0.3)	5.2 (1.0)	7.9 (2.7)	12.1 (2.1)
4 weeks	3.6 (0.3)	2.5 (0.2)	2.7 (0.2)	2.1 (0.3)	6.1 (0.3)	11.6 (3.9)	16.4 (0.7)
12 weeks	5.9 (0.5)	5.0 (0.3)	5.0 (0.7)	4.1 (0.5)	9.7 (0.3)	32.2 (7.4)	129.5 (29.3)[1]
Direct UHT; 25°C							
4 weeks	4.4 (0.8)	3.4 (0.4)	3.7 (0.1)	3.2 (0.2)	6.5 (0.4)	22.3 (2.2)	70.3 (6.2)
12 weeks	5.9 (0.3)	3.1 (0.2)	4.2 (0.4)	3.7 (0.7)	6.5 (0.3)	50.5 (9.5)	190.3 (9.6)[1]
Indirect UHT; 4°C							
1 week	2.1 (0.6)	2.6 (1.2)	1.6 (0.1)	1.7 (0.5)	1.9 (0.3)	1.3 (0.6)	13.5 (2.9)
4 weeks	2.6 (1.1)	1.8 (0.8)	1.3 (0.6)	1.5 (0.7)	1.6 (0.7)	1.3 (0.5)	303.5 (31.8)
12 weeks	4.1 (1.4)	4.8 (0.8)	2.5 (0.7)	3.4 (0.8)	2.1 (0.9)	2.7 (0.6)	320.2 (12.2)
Indirect UHT; 25°C							
4 weeks	5.2 (0.9)	5.5 (1.6)	4.8 (0.3)	3.5 (0.5)	3.7 (0.6)	3.1 (0.5)	198.6 (11.8)[1]
12 weeks	5.9 (1.8)	5.1 (1.5)	3.5 (1.9)	1.1 (0.3)	5.2 (1.9)	5.4 (1.3)	210.8 (21.9)[1]

[1]: In these milk products, sedimentation was so great that weak, gel-like consistencies, with phase separation occurred. The mass of sediment was estimated after decantation of the supernatant.

casein micelles more than offset the tendency of a reduced apparent viscosity to increase the rate of sedimentation.

In UHT milk products with FWP, sediments exhibited viscous, semi-solid, custard-like consistencies and occupied from ~ 1/3 - 2/3 of the milk volume. In the most unstable milk products, phase separation occurred; the sediment was submerged beneath a greenish, translucent, whey-like layer of liquid. The composition of sediment obtained from 12 week old F3 milk is shown in Table VI-10. The high concentrations of protein, ash, calcium and phosphorus in the sediments suggest that the low colloidal stability of the protein was related to an increased concentration of minerals. In the products with phase separation, it appeared that the entire casein component destabilized with resultant entrapment of fat globules; the translucent nature of the supernatant indicated the absence of light scattering particles.

Table VI-10. Mean (n = 3) composition, determined after 12 weeks storage at 25°C, of sediments obtained from protein-standardized, direct or indirect UHT milk products, where F3 indicates milk with the highest level of added fermented whey UF permeate. Numbers in brackets are the standard deviations.

Milk product	*F3 milk; direct UHT*	*F3 milk; indirect UHT*
g 100 g^{-1}		
Total solids	15.94 (0.47)	14.44 (0.43)
Protein	5.00 (0.10)	8.93 (0.09)
Fat	1.15 (0.09)	3.88 (0.34)
Lactose	4.64 (0.20)	4.52 (0.19)
Ash	1.52 (0.03)	1.00 (0.02)
mg 100 g^{-1}		
Calcium	365 (15)	194 (7)
Phosphorus	226 (1.6)	127 (0.7)

Two basic possibilities, or possibly their combination, ensue as to the adverse effect of extra salts on the colloidal stability of milk proteins in UHT milk: (1) Although neutralization to pH 6.8 allowed milk with FWP to be UHT-heated without visible coagulation, the presence of extra minerals may have promoted protein aggregation, on a microscopic scale, during heating; such aggregation may have manifested itself as an increased rate of sedimentation during subsequent storage. (2) Extra minerals promoted association between casein micelles during storage, leading to a faster rate of sedimentation.

It seems likely that the presence of extra soluble minerals in the FWP milk products led to limited protein aggregation during heating at pH 6.8. Protein coagulation was rapid when milk with the lowest level of FWP was direct UHT-heated at its unaltered pH (6.65), which was quite close to the neutralized milk pH of 6.8. Interactions between casein micelles might also occur upon storage and the presence of extra minerals could have a similar effect on these interactions, though the rate of collision would be reduced at 4 or 25°C, compared to the high temperature of UHT heating.

It is generally accepted that age-gelation of UHT milk involves aggregation of casein micelles into a three-dimensional network (Harwalkar, 1992). It is thought that the micelles develop an increased propensity to undergo association, due to alterations of micelle structure at the surface; it is not certain if these changes are related to non-enzymatic, physico-chemical changes or are caused by the action of proteinases, such as plasmin or bacterial proteinases (Harwalkar, 1992). Either way, it is clear that the casein in UHT milk becomes more sensitive to precipitation by calcium upon storage (Nakai *et al.*, 1964; Samel *et al.*, 1971). In the present study, the increased ratio of soluble calcium to protein in the FWP milk products might be expected to hasten the onset of age-gelation. However, age-gelation is characterized by the development of a continuous network of casein micelles to form a thixotrophic gel, without phase separation. Furthermore, age-gelation is characterized by a sudden and large increase in apparent viscosity, rather than a more gradual process observed in the present study. Therefore, the destabilization of UHT milk with FWP was probably related to a greater rate of sedimentation, rather than age-gelation.

It was clear that the sediments obtained from UHT milk products with FWP did not resemble an isoelectric precipitate of casein; unlike the case of an isoelectric precipitate, the sediments were readily redispersed upon shaking, whereupon the appearance and texture of the milk resembled that of normal UHT milk.

Because the storage stabilities of the UHT milk products were assessed over a 12 week time frame, the impact of permeate addition on the age-gelation process could not be determined, considering that age-gelation requires 6 - 12 months storage. However, the results obtained here may still be of practical merit, considering that the maximum storage time for UHT milk in Switzerland and probably other countries is about 12 weeks.

Sensory quality

The results of the sensory evaluations of the UHT milk products are shown in Table VI-11, from which the following trends are noted: (1) Direct UHT milk products had higher sensory scores than indirect UHT products. (2) Storage of milk at 25°C caused a gradual decline in sensory score, while at 4°C the sensory score was almost unchanged. (3) Milk with all three levels of added SMP had a sensory score similar to control milk, over storage at 4 or 25°C for up to 12 weeks. (4) Standardization of UHT milk with FWP reduced the sensory score, especially in the case of indirect UHT milk products.

The flavour of UHT milk is often described as being initially 'cooked', becoming 'stale' or 'oxidized' over storage (Nursten, 1995; Andersson and Öste 1995b). Cooked flavour is due to the heat-induced activation of sulphydryl groups of proteins, especially β-lactoglobulin and bovine serum albumin; activation of sulphydryl groups may lead to the formation of volatile sulphur-containing compounds, including hydrogen sulphide, methanethiol, dimethyl sulphide and dimethyldisulphide. These compounds have been associated frequently with a cooked flavour (Steely, 1994), though the influence of numerous other heat-generated compounds on flavour is relatively unknown (Badings and Neeter, 1980; Nursten, 1995; Andersson and Öste, 1995b; Bosset *et al.*, 1996). The flavour of control milk or milk standardized with SMP, after 1 week storage at 4°C, was described as cooked, flavour intensity being greater in the indirect UHT milk products. Andersson and Öste (1992) demonstrated that the higher heat load used during the manufacture of the indirect UHT milk leads to a more intense cooked flavour, due to greater denaturation of whey proteins and thiol group reactivity.

Although the overall sensory scores of control milk or milk containing SMP were similar, a number of panelists detected a less intense cooked flavour in the standardized milk samples; this effect was most pronounced after 1 week storage, when the cooked flavour of all milk products was most intense. A speculative explanation for this could be that the presence of SMP increased the dissolved oxygen content of the milk; the dissolved oxygen content of the permeate was not measured, but could have been high if conditions during UF were turbulent. The presence of oxygen leads to a disappearance of the cooked flavour of UHT milk, which has been correlated with the oxidation of free sulphydryl groups (Thomas *et al.*, 1975; Fink and Kessler, 1986; Andersson and Öste, 1992).

Table VI-11. Influence of protein standardization, type of UHT heat treatment and temperature of storage on the mean sensory score of milk. The terms S1, S2 or S3 indicate milk standardized with increasing amounts of skim milk UF permeate; similarly, F1, F2 or F3 denote milk with increasing quantities of fermented acid whey UF permeate. Bracketed numbers are the standard deviation of each data point.

Heat treatment; storage temperature	*Control milk*	*S1 milk*	*S2 milk*	*S3 milk*	*F1 milk*	*F2 milk*	*F3 milk*
	Hedonic sensory score						
Direct UHT; 4°C							
1 week	8.4 (1.9)	8.3 (1.7)	8.5 (1.7)	6.5 (2.3)	7.4 (1.9)	7.9 (1.6)	5.7 (1.8)
4 weeks	9.1 (2.0)	8.6 (1.0)	8.6 (1.0)	8.3 (1.4)	7.9 (1.2)	6.9 (2.0)	5.2 (1.0)
Direct UHT; 25°C							
4 weeks	6.8 (1.7)	6.7 (1.2)	6.5 (1.1)	6.4 (1.1)	4.8 (1.9)	4.6 (1.2)	4.1 (1.3)
12 weeks	6.6 (2.2)	6.4 (2.0)	6.0 (2.1)	6.1 (1.1)	4.6 (1.0)	4.4 (0.5)	3.6 (1.2)[1]
Indirect UHT; 4°C							
1 week	8.6 (1.3)	7.8 (1.7)	7.2 (1.1)	7.4 (1.8)	7.5 (1.9)	6.0 (1.5)[1]	3.1 (1.2)[1]
4 weeks	6.8 (1.1)	7.2 (2.1)	7.2 (0.8)	7.2 (1.4)	7.2 (2.1)	6.0 (1.5)[1]	3.0 (1.0)[1]
Indirect UHT; 25°C							
4 weeks	7.2 (1.4)	5.6 (3.2)	7.1 (2.3)	6.6 (1.1)	6.9 (1.9)	5.6 (1.0)	3.4 (0.6)[1]
12 weeks	7.0 (1.4)	7.5 (1.6)	7.1 (1.7)	7.0 (1.0)	6.6 (1.9)	5.4 (0.8)	3.0 (0.5)[1]

[1]: These samples were shaken thoroughly to disperse the large amounts of sediment prior to sensory analysis.

Storage of control milk or milk products containing SMP led to a disappearance of the cooked flavour; milk products became blander and developed stale or oxidized flavours, which reduced the hedonic sensory score. The development of oxidized flavour in UHT milk during storage has been attributed to the auto-oxidation of lipids during storage and Maillard browning to generate a variety of aldehydes and ketones (Jeon *et al.*, 1978; Andersson and Öste, 1995b). Probably, the reactions responsible for the development of stale flavour would proceed at a faster rate at 25°C than at 4°C, leading to the lower sensory quality of milk products stored at 25°C.

Milk standardized with FWP also displayed, initially, a cooked flavour, which became oxidized during storage. However, these flavours were difficult to detect because of the presence of strong off-flavours, described by panelists as being 'unclean', 'unnatural', ' fermented' or 'acidic'. This agrees with the observations of Chapter IV, where it was reported that the sensory quality of pasteurized skim milk was reduced drastically when standardized with a FWP, which was attributed to low molecular weight compounds produced by lactococcal fermentation of lactose. During direct UHT heating, vacuum flash cooling of the milk may have led to volatilization and loss of some of the off-flavours present in the FWP; this was probably responsible for the higher sensory score of direct UHT milk, compared to indirect UHT milk. This improvement in sensory quality was modest and direct UHT milk products with FWP were still deemed unacceptable for consumption.

As described above, some of the UHT milk products with FWP underwent extremely high sedimentation upon storage resulting in phase separation. Prior to sensory analysis, these samples were shaken to disperse the gel-like material to obtain milk with a uniform appearance and texture. Had this procedure not been carried out, panelists would have been presented with a gel-like material submerged beneath a greenish liquid - without doubt an extremely unappealing product and completely inconsistent with the normal visual attributes of UHT milk. Therefore, the negative sensory impression of UHT milk products with FWP would have been much greater if their unaltered appearance had been considered.

Conclusions

Milk with protein content 'down'-standardized by the addition of SMP was equivalent to or possibly even more suitable than normal milk for direct or indirect UHT processing, with respect to heat stability, storage stability and sensory quality; nutritional quality was reduced slightly, due to a reduced content of protein, calcium, phosphorus and potassium, but changes in other micronutrients, such as magnesium, sodium, chloride and vitamins A, B_1, B_2 and E were negligible. The 'downward' standardization of milk with FWP improved the complement of micronutrients, compared to the use of SMP; changes in calcium and phosphorus were almost negligible. In other respects the use of FWP was highly unsatisfactory; neutralization to pH 6.8 was required to avoid coagulation during UHT heating, products underwent excessive sedimentation, developed high viscosities during storage and possessed unacceptably strong off-flavours.

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- Chapter VII -

Influence of protein standardization and ultra-high temperature heating on the furosine value and freezing point of milk*

Introduction

A number of methods exist to assess the severity of heat treatments applied to milk and other dairy products (Pellegrino *et al.*, 1995). These include the measurement of inactivation of indigenous milk enzymes, whey protein denaturation or loss of its solubility, or determination of the concentration of heat-formed compounds in milk, such as lactulose or products of the Maillard reaction (MR). The manufacture of ultra-high temperature (UHT) milk involves relatively severe heat treatments, causing lactose to undergo a complex network of reactions, including its isomerization, degradation, and reaction with ε-amino groups of proteins in the initial stages of the MR (Berg, 1993).

Lactuloselysine (galactosefructoselysine) is an Amadori compound, generated by the reaction of a reducing sugar (lactose) with a free ε-amino group of lysine, and is found in milk, during the early stages of the MR. The concentration of lactuloselysine in milk may be determined indirectly after its acid hydrolysis into pyridosine and furosine and measuring the furosine value (FV) by high pressure liquid chromatography (HPLC). The recent development of rapid HPLC methods to measure furosine (Resmini *et al.*, 1990; Henle *et al.*, 1991; Resmini and Pellegrino, 1991), have stimulated interest in the use of FV as a heat load indicator.

The principal factors affecting the formation of lactuloselysine in milk are the concentration of reactants (lactose and lysine) and fat, the heat load applied and the temperature at which the heat-treated milk is stored (Pellegrino, 1994). This multiplicity of factors complicates the correlation of FV with heat load; the FV can vary widely for milk products belonging to the same heat class (Pellegrino *et al.*, 1995). It has been recommended that FV be reported as mg furosine per mg protein to allow a more direct correlation of FV to heat load, but this approach ignores the effect of possible variations in the fat and lactose content of milk and the effect of storage conditions.

The freezing point (FP) of unheated milk exhibits very little natural variation, but the influence of heat processing on the FP is less well known; if heating caused the

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concentration of low molecular weight entities to change, then the FP should change. Heating of milk initiates a complex network of reactions, including reactions of lactose (Berg, 1993) and changes in the partitioning of salts between the soluble and colloidal states (Holt, 1995), but the influence of these changes on the FP of milk has apparently not been determined.

In the previous Chapter, the physical, chemical and sensory properties of protein 'down'-standardized UHT milk were characterized extensively; the standardization was carried out using permeate derived by ultrafiltration (UF) of skim milk (SMP) or quarg acid whey (FWP). For the same milk products, measurements indicating the effect of protein standardization and UHT heating on the FV and FP of milk were also carried out, the results of which are reported here. Particular objectives of the present study were: (1) To establish whether changes in the concentration of protein and lactose caused by protein standardization would interfere with the use of FV as an index of direct or indirect UHT heat treatment; and (2) To determine the effect of protein standardization and UHT heating on the FP of milk, more specifically, to determine if UHT heating would cause an irreversible change in milk salt equilibria and/or free lactose concentration, which could be detected by measurement of FP.

Materials and methods

Preparation of protein-standardized, ultra-high temperature milk

Normal and protein-standardized, UHT heated milk products were the same products as in Chapter VI. All milk products contained ~ 1.7% fat and ~ 3.4 (control milk), 3.2, 2.9 or 2.6% protein; the 'down' standardization was accomplished by using permeates derived by UF of skim milk (SMP) or quarg acid whey (FWP). The milk products were stored, in darkness, at 4 or 25°C, for 12 weeks. Like in Chapter VI, milk products containing SMP are denoted as S1, S2 or S3 milk, in order of increasing level of added SMP or decreasing protein content; similarly, milk products with FWP are termed F1, F2 or F3 milk.

Compositional analyses

In discussions on the FV and FP, it is relevant to refer to the protein and lactose contents of the milk samples, which were exactly the same as shown in Table VI-1.

Determination of furosine value

The FV's of UHT milk products were determined by the method of Resmini *et al.* (1990). This involved acid hydrolysis of lactuloselysine into pyridosine and furosine and quantification of the furosine by HPLC. For calibration of the HPLC, 3 mg of furosine (acquired from Neosystem Laboratorie, Strasbourg, France) was dissolved in 100 mL of 0.3 M HCl and 10 mL of that solution was then dissolved in 90 mL of 0.3 M HCl. The FV was expressed as mg furosine L^{-1} of milk. For each milk, the FV was determined in duplicate, on three separate occasions, and mean and standard deviation values calculated using all data (n = 6).

Measurement of freezing point

The FP of unheated SMP or FWP and unheated or UHT-heated milk samples, was measured in °C by the cryoscopic FP determination method (AOAC, 1990), the principle of which is described in section 2.4.2. A Fiske MS Cryoscope (Fiske Associates, Uxbridge, MA) was used and measurement of FP was carried out in duplicate, on three separate occasions, and mean values and standard deviations were calculated from all data (n = 6).

Results and discussion

Furosine value

The effect of protein standardization, type of UHT heating and storage temperature on the FV of UHT milk is shown in Table VII-1; the relative importance of these factors on the FV is summarized in Table VII-2.

The FV was much higher in the indirect UHT milk, compared to the direct UHT milk, when all products were 1 week old, which could have been due to two effects: (1) The higher total heat load applied during indirect UHT treatment (Nangpal *et al.*, 1990a); (2) The effect of temporary dilution, caused by steam infusion during direct UHT treatment, on MR kinetics (Nangpal *et al.*, 1990b). The non-instantaneous nature of heating and cooling and the more intense pre-heating of the milk in indirect UHT heating, causes milk to experience a greater heat load, compared to direct UHT heating. This would increase the rate of formation of lactuloselysine; collision frequency between reactants would be increased and a greater number of molecules would have energies equal to the activation energy (ΔE^*).

Table VII-1. Influence of protein standardization, type of UHT heat treatment and temperature of storage on the mean furosine value of milk. The terms S1, S2 or S3 indicate milk standardized with increasing amounts of skim milk UF permeate; similarly, F1, F2 or F3 denote milk with increasing quantities of fermented acid whey UF permeate. Bracketed numbers are the standard deviation of each data point.

Heat treatment; storage temperature	*Control milk*	*S1 milk*	*S2 milk*	*S3 milk*	*F1 milk*	*F2 milk*	*F3 milk*
	Furosine value (mg L^{-1})						
Direct UHT; 4°C							
1 week	11.5 (0.2)	11.2 (0.6)	10.8 (0.5)	9.1 (0.5)	11.1 (1.6)	9.1 (0.6)	8.5 (0.7)
4 weeks	12.9 (1.9)	12.0 (1.0)	10.4 (0.8)	9.2 (0.0)	10.4 (0.1)	10.0 (0.1)	8.1 (0.1)
12 weeks	13.2 (0.5)	11.7 (0.6)	12.0 (1.4)	11.1 (0.8)	12.6 (0.9)	9.9 (0.1)	10.4 (0.1)
Direct UHT; 25°C							
4 weeks	27.8 (0.8)	25.6 (0.6)	23.2 (0.2)	19.3 (0.5)	21.9 (.16)	22.3 (0.1)	15.1 (0.1)
12 weeks	53.3 (8.8)	43.9 (0.1)	39.4 (0.1)	36.0 (0.1)	39.1 (8.1)	36.2 (1.7)	36.9 (5.4)
Indirect UHT; 4°C							
1 week	46.8 (5.8)	32.0 (1.7)	29.7 (2.4)	26.9 (5.1)	35.1 (1.7)	28.2 (1.1)	17.5 (1.1)
4 weeks	43.4 (1.4)	40.2 (3.5)	33.3 (0.8)	25.7 (1.6)	37.0 (1.8)	35.4 (0.1)	20.7 (0.1)
12 weeks	42.0 (3.7)	36.3 (5.8)	29.7 (0.5)	25.7 (0.9)	30.6 (6.6)	27.7 (0.6)	17.0 (1.7)
Indirect UHT; 25°C							
4 weeks	50.6 (4.3)	45.9 (4.4)	41.7 (1.9)	33.4 (0.1)	39.7 (0.1)	31.7 (0.1)	20.2 (0.1)
12 weeks	66.7 (3.8)	58.7 (0.1)	58.0 (4.8)	58.6 (7.4)	56.6 (1.9)	51.7 (3.0)	35.2 (0.1)

Table VII-2. Relative influence of protein standardization with skim milk UF permeate (SMP) or fermented whey UF permeate (FWP), type of UHT heat treatment and temperature of storage on the furosine value (FV) of milk.

Factor	*Effect on FV*[1]	*Effect on FV related to:*
Protein standardization:		
With SMP	↓	reduced concentration of protein
With FWP	↓↓	reduced concentration of protein and lactose
UHT heating:		
direct	↑↑↑	heat load; temporary dilution of reactants
indirect	↑↑↑↑	heat load
Storage:		
at 4°C	none	no Maillard reaction
at 25°C	↑↑↑	slow Maillard reaction

[1]: Effect was to increase the furosine value by a relatively large (↑↑↑) or very large (↑↑↑↑) extent or to reduce the furosine value by a relatively slight (↓) or moderate (↓↓) extent.

During direct UHT heat treatment, the dilution of reactants coincides with the maximum temperature of heating, which has been conjectured to have a considerable effect on the rate of formation of lactulose and lactuloselysine. From experimental data, Nangpal *et al.* (1990b) showed that for the same heat load, increasing the concentration of lactose in milk led to greater formation of lactulose and furosine, and argued, by calculation, that this was caused by a concomitant increase in the pre-exponential factor (k_0) of the Arrhenius equation:

$$k = k_0 \exp(-\Delta E^*/RT),$$

where k is the rate constant, R the universal gas constant and T is the absolute temperature.

The hypothesis of Nangpal *et al.* (1990b) should be treated with caution. To simplify their calculations, the authors assumed that the formation of furosine approximately obeyed zero order kinetics, yet at the same time suggested that k_0 and hence the reaction rate were functions of concentration; these two postulates are mutually exclusive. In a systematic study, Berg (1993) discussed the complexity of the thermal reactions of lactose in milk and stressed the importance of considering the complete matrix of reactions in order to calculate reliable kinetic parameters. The use of the Arrhenius

equation to elucidate chemical kinetics can lead to oversimplification; the Eyring equation, developed from the activated complex theory of chemical reactions, has been recommended as a less empirical approach to describing kinetics (see van Boekel and Walstra, 1995).

The presence of increasing amounts of either SMP or FWP in UHT milk caused the FV to decline and the effect was evident throughout storage, but it was too small to invalidate the use of FV as an indicator of direct or indirect UHT heat treatment. For example, the 7 direct UHT milk products stored for 1 week at 4°C had FV's in the range 11.5 - 8.5 mg L^{-1}; for indirect UHT milk the range was 46 - 17.5 mg L^{-1}; since these values did not overlap, differences in protein and lactose concentration due to standardization did not lead to ambiguity in the use of the FV as a heat load indicator. This reduction in FV caused by standardization was expected, because the presence of increasing amounts of SMP caused a progressive decline in protein concentration, while the use of FWP caused protein and also lactose concentration to decline (Table VI-1); lactose and protein are the reactants involved in the formation of lactuloselysine. The decline of FV was greater in the indirect than in direct UHT milk products. It is conceivable that the more severe heating employed in the manufacture of indirect UHT milk accentuated the influence of initial reactant concentration on the formation of lactuloselysine.

Storage of direct or indirect UHT milk, at 25°C, caused a gradual increase in the FV, while at 4°C changes in the FV were negligible. After 12 weeks at 25°C, the average increase of FV in all products was ~ 18 mg L^{-1}, which is of a similar magnitude to the FV increases of ~ 15 mg L^{-1} after 6 weeks storage of UHT milk at 30°C, reported by Nangpal and Reuter (1990). The increase in FV at 25°C was probably caused by a slow MR, which would not occur at 4°C. In some cases, the increase was so marked, that if storage conditions were unknown, it would not be possible to distinguish with confidence between direct and indirect UHT milk; when any direct UHT milk was stored at 25°C for 12 weeks, the FV was greater than that in indirect UHT milk stored at 4°C for 1, 4, or 12 weeks.

Although trends in the FV were established clearly, the results displayed a certain degree of variability. This variability could be attributed to the reproducibility of the sample preparation techniques and precision of the method used to assay for furosine or, possibly, to variations in the fat content of the milk samples; the target fat content was 1.7%, but deviations up to 10% form this values were measured (Table VI-1). Pellegrino (1994) established that, during direct or indirect UHT heating of milk, fat inhibited the formation

of lactulose and lactuloselysine, probably because it increased the apparent viscosity, which would slow down diffusion and collision of reactants.

Freezing point

The mean FP of 1.7% fat milk, with 3.36% protein was - 0.525 °C, typical for that of normal milk. The FP values of SMP or AWP were - 0.484 °C or - 0.654 °C, respectively, and addition of these permeates to milk led to progressively lower or higher FP values (Table VII-3), confirming the trends reported in Chapter IV.

Table VII-3. Mean (n = 6) freezing point, measured before UHT heating or after 1 week of storage at 4°C, of control milk and protein-standardized UHT milk products, where S1, S2 or S3 indicate milk with increasing amounts of skim milk UF permeate and F1, F2 or F3 denote milk with increasing quantities of fermented whey UF permeate. Numbers in brackets are the standard deviations.

Product	*Before heating*	*After UHT direct*	*After UHT indirect*
	Freezing point (°C)		
Control milk	- 0.527 (0.005)	- 0.527 (0.002)	- 0.521 (0.005)
S1 milk	- 0.524 (0.002)	- 0.524 (0.002)	- 0.520 (0.002)
S2 milk	- 0.521 (0.004)	- 0.523 (0.001)	- 0.517 (0.003)
S3 milk	- 0.517 (0.006)	- 0.517 (0.006)	- 0.518 (0.001)
F1 milk	- 0.545 (0.019)	- 0.535 (0.001)	- 0.532 (0.002)
F2 milk	- 0.557 (0.007)	- 0.553 (0.003)	- 0.551 (0.001)
F3 milk	- 0.573 (0.002)	- 0.573 (0.001)	- 0.571 (0.002)

The high FP of the SMP was due to its low ash content (Table VI-1), which was also reported in Chapter IV. The mineral content of the FWP was similar to that of normal milk, but because much of the salts in milk are associated with casein they would be expected not to contribute to the FP depression, whereas the salts in the FWP must have been completely soluble and hence led to a greater depression of FP. Milk with FWP had a lower content of lactose than normal milk, but a greater amount of organic acids, evident by the greater titratable acidity of the FWP (65 °Th), compared to normal milk (16 °Th); the greater content of organic acids probably compensated for the tendency of a reduced concentration of lactose to increase the FP.

When Table VII-3 is compared to Figure IV-1, it can be seen that in the present case the addition of SMP to milk for protein standardization caused a lesser increase in the FP, compared to that reported in Chapter IV; this was expected as the FP of the SMP in Chapter IV was - 0.465°C, while here the FP of - 0.484°C for SMP was closer to that of normal milk. In the present case, when the protein content of milk was standardized 'down' to 2.55% protein (S3 milk, Table VI-1), the FP was - 0.517°C, whereas in Chapter IV, at ~ 2.6% protein in milk, the FP was near - 0.508°C (a FP ≥ - 0.508°C is regarded as proof of the presence of extraneous water in milk). The results here indicate that standardization of milk with SMP is possible without substantial changes in the FP, when the SMP is made under typical industrial UF conditions; the unusual conditions during the UF of skim milk were discussed in Chapter III.

When milk is heated, it undergoes a complex matrix of reactions, including isomerization and degradation of lactose and the reaction of lactose with lysine (Berg, 1993), changes in salt equilibria (Holt, 1995), dephosphorylation of casein (Dalgleish *et al.*, 1987), and denaturation and aggregation of proteins (McCrae and Muir, 1995); the effects of these changes on the colligative properties of milk, including FP, has not received much attention. The FP of control or protein-standardized milk products after direct or indirect UHT heating was virtually unchanged, indicating that the heating process did not change the overall molality of milk or, if it did, the change was reversible during storage at 4°C. During heating of milk, association of salts with casein micelles increases, but the shift is reported to be reversible upon cooling, the rate of re-equilibration decreasing with increasing severity of heat treatment (Geerts *et al.*, 1983; see section 2.3.2, Chapter II). When milk was heated at 85°C for 40 min and cooled to 4°C for 24 h, almost all of the calcium and phosphorus that had been heat-precipitated reverted to the native state (Pouliot *et al.* 1989). Accordingly, in the present study, the storage of direct UHT milk for 1 week at 4°C, would be expected to provide ample time for milk salts to return to their normal state, after a heat-induced shift in salt equilibria.

Data on the FV indicate that during heating the MR took place in the direct and more especially indirect UHT milk products (Table VII-1), but this did not have a noticeable effect on FP. The MR and other reactions of lactose are complicated (Berg, 1993) and attempting to predict their influence on the colligative properties of milk would be difficult. However, any possible effect of the MR would be extremely small, considering that the

concentration of lactose in the UHT milk products was ~ 1000 - 5000 times greater than the concentration of furosine. Thus, the proportion of lactose involved in the MR and probably other thermal reactions was negligible and any accompanying changes in molality were too small to noticeably affect the FP of milk.

Conclusions

The FV of freshly-processed, indirect UHT milk was consistently greater than that in direct UHT milk, despite fluctuations in the concentration of the reactants, lactose (~ 4.8 - 5.0%) and protein (~ 2.6 - 3.4%), involved in the heat-induced formation of furosine. After 12 weeks at 25°C, some direct UHT milk products had a greater FV than indirect UHT milk products stored at 4°C for 1 - 12 weeks. Therefore, inadequate knowledge of storage conditions of UHT milk could have led to incorrect correlation of FV with the type of UHT heat treatment, while fluctuations in the concentration of protein and lactose caused by protein standardization were of lesser importance.

The FP of normal milk or any protein-standardized milk was not altered after direct or indirect UHT heat treatment, when the FP of UHT milk was measured after 1 week storage at 4°C. This indicates that any changes in the partitioning of milk salts which may have been caused by direct or indirect UHT heating were reversible. Although UHT heating and storage at 25°C caused the MR to proceed, this had no effect on the FP of milk.

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- Chapter VIII -

Thermal stability of whey protein solution/skim milk blends*

Introduction

The issue of protein standardization has recently received much attention by the International Dairy Federation, but as discussed in section 5 of Chapter II, more drastic alteration of the protein fraction of milk, termed 'protein adjustment', could also lead to a number of benefits. These include the protein adjustment of fluid milk in order to modify the properties of dairy products manufactured from such milk, such as yoghurt or cheese. The recent investigations of Wong and Watson (1995) and McIntosh *et al.* (1995), which showed the anticarcinogenic properties of whey proteins, may provide a further incentive for protein adjustment, by increasing the ratio of whey protein to casein protein in milk.

The influence of protein adjustment on the physico-chemical properties of milk, including that of heat stability, has received limited attention. In Chapter V, it was shown that protein adjustment of milk, by the direct addition of a fraction of α-la, caused a modest decline in heat stability, when measured at the unadjusted pH (~ 6.7) of milk. Patocka *et al.* (1993) ultrafiltered different types of whey to obtain UF retentate with a protein content similar to that of skim milk. The UF retentate was mixed with skim milk to obtain different ratios of whey protein to casein while maintaining constant total protein. When the UF retentate was derived from sweet or ultracentrifugal whey, blends of skim milk and UF retentate had heat stabilities, measured at 90°C and pH 6.5 - 6.7, in excess of 30 min. In contrast, mixtures of acid whey UF retentate and skim milk, adjusted to pH 6.5 - 6.7, were heat labile at ratios > 30/70, due to the high calcium content of the acid whey UF retentate, which led to calcium-modulated precipitation of whey proteins and casein. Similar trends were found by Abd El-Salam *et al.* (1991), who reported that a mixture of liquid whey protein concentrate (WPC) and buffalo milk, at ratios > 30/70, coagulated rapidly at 80°C. In these studies heat stability was measured at a limited range of pH values and temperature; and no attempt was made to determine the relative importance of specific milk proteins on heat stability.

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The effects of whey proteins on the heat stability of model solutions of casein have been studied by a number of authors (Fox and Hoynes, 1975; Fox and Hearn, 1978; Kudo, 1980; Singh and Fox, 1985, 1986, 1987a, b). A general conclusion of these studies is that α-la and β-lactoglobulin (β-lg) increase the resistance of casein micelles to thermal coagulation at < pH 6.7, but impart heat sensitivity at > pH 6.7. At < pH 6.7, thermal association of whey proteins onto casein micelles may occur, increasing their charge and hydration and thus heat stability; at > pH 6.7, the reduced heat stability may be caused by a rapid loss of κ-casein from the surface of micelles, via complexation with whey proteins.

In this study, dried, industrially-produced, fractions of α-la (α-F), β-lg (β-F) or a WPC were dissolved in skim milk UF permeate (SMP) and the solutions (α-FS, β-FS or WPCS) blended with skim milk, to increase the ratio of whey protein to casein without changing the concentration of total protein. The pH of the blends was adjusted to values in the range 6.4 - 7.1 and the heat coagulation time (HCT) determined at 140°C.

Materials and methods

Materials

Fractions of α-la, β-lg or a WPC used in this study were all produced on an industrial scale. The α-F and β-F were provided by Protose Separations Inc., Toronto, Ontario, Canada; the manufacturing procedure was not disclosed. The WPC was 'Alacen' brand # 132, obtained from New Zealand Milk Products, Inc., Santa Rosa, California; details of product manufacture were not provided. The α-F, β-F or the WPC were dissolved in SMP at 3.44% (w/w) protein and each solution blended with skim milk (3.44%, w/w, protein) at ratios of 100/0 - 40/60 (g skim milk/g whey protein solution), so that the ratio of whey protein to casein protein was increased, but total protein was constant. The SMP was made by UF of skim milk, using the Carbosep UF system, as described in Chapter III. In some experiments, the heat stabilities of solutions of α-F, β-F or the WPC were determined in the absence of casein, at temperatures of 96 - 140°C, using the HCT test method. For this purpose the protein preparation was dissolved in SMP at 1.7% (w/w) protein.

Analyses

All raw materials were assayed for total solids, protein and ash by routine analytical methods (AOAC, 1990). The heterogeneity of the protein in the α-F, β-F or WPC was determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using the same separation, staining and destaining procedures, as described in Chapter V.

Heat stability

All heat stabilities were measured using the HCT test method, used also in Chapter III. The HCT values were determined in triplicate or quadruplicate, from separately prepared protein solutions, and mean values calculated from all data.

The behaviour of specific proteins during heating of the skim milk/whey protein solution mixtures was investigated further by SDS-PAGE. An aliquot (10 mL) of required sample was placed in a glass tube (internal diameter 14 mm), the tube sealed and immersed in a 120°C oil bath for 5 min; this milder heat treatment was used in an attempt to simulate conditions during the early stages of the heat coagulation process and cause aggregation of the most labile proteins. The sample was cooled quickly to 20°C and centrifuged at 20°C and 100 000 x g for 1 h, using a Beckman Ultracentrifuge (model L8-70M, Beckman Instruments Inc., Palo Alto, CA). The purpose of centrifugation was to sediment the most heat sensitive whey proteins, which would be expected to heat-aggregate independently and/or complexed to casein micelles. A portion (10 ± 0.5 mg) of ultracentrifugal (UC) pellet was dissolved in 1 mL of the same SDS reducing buffer used in Chapter V; 5 μL of this solution was applied to a 12.5% SDS gel and the protein was separated, stained and destained using the procedure described in Chapter V.

Results and discussion

Composition of raw materials

The composition of skim milk and SMP has already been described in Chapter III. In this study, the compositions of independently acquired samples of skim milk and SMP were determined; the results, which are shown in Table VIII-1, are very similar to those shown in Table III-1. Additional compositional data shown here indicated that the α-F and β-F were high in protein and low in lactose and ash.

Table VIII-1. Average composition of materials used to prepare protein-adjusted milk.

Product	*Moisture*	*Protein*[1]	*Lactose*	*Ash*
	% (w/w)			
Skim milk UF permeate	94.8	0.31	4.4	0.36
α-Fraction	4.1	89.9	1.0	2.95
β-Fraction	5.2	89.0	1.0	2.81
Whey protein concentrate	4.2	78.7	11.8	3.89

[1]: % Protein = N x 6.38

Electrophoretograms (results not shown) confirmed that α-la or β-lg were by far the most abundant proteins in the α-F or β-F, respectively; the α-F was more heterogeneous, containing also noticeable quantities of β-lg and bovine serum albumin (BSA). The WPC had a relatively high amount of non-protein constituents and a heterogeneous protein fraction, of which β-lg was the most abundant protein. When the α-F, β-F or WPC were dissolved in SMP at 3.44% protein, the final pH values were in the range 6.6 - 6.8 and changes in the pH of skim milk (6.7) were very small (± 0.05 pH units) upon admixture of any whey protein solution at any ratio.

Heat stability of solutions of α-fraction, β-fraction or whey protein concentrate

To permit a better understanding of the thermal behaviour of proteins in the skim milk/whey protein solution blends, the heat stability of individual whey protein preparations in SMP was measured at 96 - 140°C. The α-FS was extremely heat stable (Figure VIII-1), coagulating only at high temperatures and low pH values (140°C and < pH 6.7; 120°C and < pH 6.5). This was in distinct contrast to the behaviour of the β-FS or WPCS which coagulated quickly at 120°C and almost instantaneously at 140°C; these solutions were resistant to coagulation only at a relatively low temperature (96°C) and high pH (6.7 - 6.9). The heat stability results indicate that the thermal behaviour of the α-FS was in agreement with the known resistance of α-la to heat coagulation (Larson and Rolleri, 1955), while that of the β-F or WPC was dominated by β-lg, documented as being sensitive to heat precipitation (de Wit, 1981).

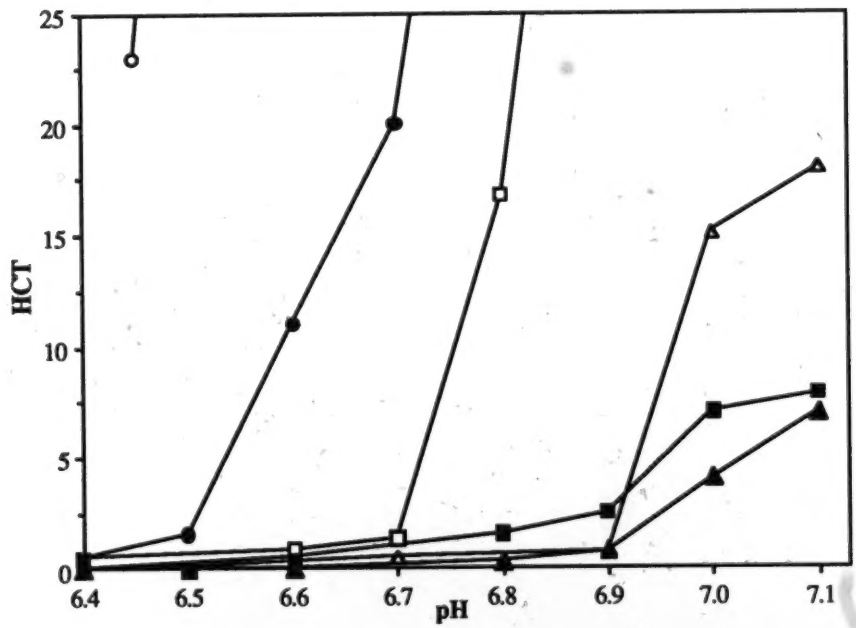

Figure VIII-1. Heat coagulation time (HCT) of pH-adjusted whey protein solutions, made by dissolving α-fraction, β-fraction or whey protein concentrate in skim milk UF permeate at 1.7% (w/w) protein; α-fraction solution at 120 (○) or 140°C (●); β-fraction solution at 96 (□) or 120°C (■); whey protein concentrate solution at 96 (△) or 120°C (▲). Lines exiting the graph indicate heat stabilities > 50 min. At 140°C and all pH values, β-fraction solution or whey protein concentrate solution had heat stabilities of ~ 0 min; at 96°C and all pH values, α-fraction solutions had a heat stabilities > 50 min.

Near neutral pH, β-lg denatures at ~ 75 - 80°C, while α-la, in the presence of calcium, denatures at ~ 60 - 65 °C (Rüegg *et al.*, 1977; de Wit *et al.*, 1983; Bernal and Jelen, 1984; Paulsson *et al.*, 1985; Paulsson and Dejmek, 1990; Xiong *et al.*, 1993; Hollar *et al.*, 1995; Anema and McKenna, 1996); in the present study both α-la and β-lg would be expected to exhibit unfolded conformations at the high temperatures of 96° - 140°C. In milk, Anema and McKenna (1996) observed that at temperatures > 85°C for α-la or > 100°C for β-lg, the proteins underwent aggregation reactions, evident by a reduction in entropy. Thus, in the heating conditions used in the present study, it can be concluded that in the ionic environment of SMP, unfolded β-lg was prone to rapid and extensive intermolecular interaction, leading to the formation of visible protein clots, whereas aggregation between unfolded α-la molecules was much more limited, such that

visible protein clots did not form. The resistance of α-la to heat coagulation reported here is consistent with the work of Chaplin and Lyster (1986), who showed that prolonged heating (100°C for up to 30 min) of α-la, in 0.1 M phosphate buffer at pH 7.0, led to irreversible denaturation and polymerization, but the extent of polymerization was very limited, allowing α-la to remain soluble. The limited propensity of α-la for thermal association was also demonstrated by Paulsson *et al.* (1986), who could not gel a 20% solution of α-la, upon heating at 90°C and pH 6.6, in contrast to β-lg which gelled at 5% protein. Hines and Foegeding (1993) observed a similar trend; when a 7% α-la solution, at pH 7 in the presence of 100 mM NaCl, was heated at 80°C for 3 h, protein aggregation was extremely slow resulting in the formation of a very weak gel. The resistance of α-la to thermal coagulation can sometimes be attributed to its calcium-assisted renaturation on cooling (Rüegg *et al.*, 1977; Bernal and Jelen, 1984; Patocka *et al.*, 1987), but continuous heating at 140°C would not allow renaturation.

Vanderheeren and Hanssens (1994) showed that binding of the hydrophobic probe, bis-ANS (1, 1'-bi [4-anilo] naphthalene-5, 5'-disulphonate) to apo-α-la, at pH 7.5, was maximal at 25°C; two strong binding sites were identified. At 25°C and pH 7.5, the protein probably existed in the molten globule state, corresponding to a loosening of tertiary structure with preservation of secondary structure, which allowed bis-ANS to more easily penetrate the hydrophobic interior of the molecule. At 80°C, apo-α-la exhibited an unfolded conformation; although the accessibility of hydrophobic groups to bis-ANS increased further, the loss of tertiary structure caused dissipation of hydrophobic clusters and only one weak binding site for bis-ANS was identified. Similar trends occurred when α-la was heated in the presence of calcium, although the effects took place at higher temperatures, because of stabilization of protein conformation by calcium. It has not been proven whether the reduced hydrophobic interaction between α-la and bis-ANS at high temperature could be extrapolated to imply that relatively weak hydrophobic interactions would occur between unfolded proteins, but if this were the case, it would, at least in part, explain the high resistance of α-la to thermal precipitation.

It is also possible that the limited ability of α-la for self-association, compared to β-lg, was related to a lower rate of sulphydryl group activity in α-la. Considering that β-lg contains a free thiol group, whereas α-la does not, thiol group interactions between denatured α-la molecules can be expected to be less prevalent than in the case of β-lg.

Calvo *et al.* (1993) showed that when α-la and casein micelles were heated together at 90°C for 24 min, α-la did not aggregate with itself or with casein micelles, but the presence of β-lg promoted aggregation of α-la. It was postulated that β-lg could act as a catalyst, via its free thiol group, which interacted with the sulphydryl bridges of α-la, thus allowing α-la to aggregate with itself, β-lg or κ-casein; this indicates that the presence of the free sulphydryl group of β-lg is strongly conducive to sulphydryl-disulphide interchange reactions.

At increased pH it is likely that α-la and β-lg were denatured to a greater extent, as repulsive intramolecular electrostatic forces would be expected to increase. However, the increased resistance of the α-FS, β-FS or WPCS to thermal coagulation, with increasing pH, indicate that intermolecular association between denatured protein molecules was also inhibited by increased electrostatic repulsion. Possibly, longer heating times were required to accentuate hydrophobic and thiol group interactions involved in protein aggregation or more extensive unfolding of protein was required to initiate association. The heat stability of the WPCS was lower than that of the β-FS, probably due to its higher content of minerals; small (0 - 200 mM) changes in the concentration of calcium have a great effect on the thermal aggregation of β-lg, due to the ability of calcium to form cross-bridges between proteins (Xiong *et al.*, 1993).

Heat stability of blends of skim milk and α-fraction solution

In general, at 140°C and initial pH 6.6 - 6.7, mixtures of skim milk and α-FS were more heat stable than skim milk alone, but considerably less heat stable when initial pH was in the range 6.8 - 7.1 (Figure VIII-2). An exception to this trend was the very low heat stability, at pH 6.6, of a 40/60 combination of skim milk and α-FS; this was probably due to the presence of heat labile β-lg in the α-F. Skim milk showed the well known type A HCT/pH profile, thought to be due to pH-dependent interactions between serum proteins and κ-casein (Singh and Fox, 1985, 1986, 1987a, b; see also section 4.1, Chapter II).

The nature of thermal interactions between serum proteins and casein was investigated further by SDS-PAGE of the UC pellets of heated (120°C/5 min) skim milk/α-FS samples. At pH 6.6, the increased ratio of α-FS to skim milk led to greater amounts of α-la, β-lg and BSA in the UC pellets (Figure VIII-3, columns 1 - 3). The results reflected the composition of the α-F which consisted primarily of α-la, but with smaller amounts of β-lg and BSA and implied that increasing the amount of these proteins

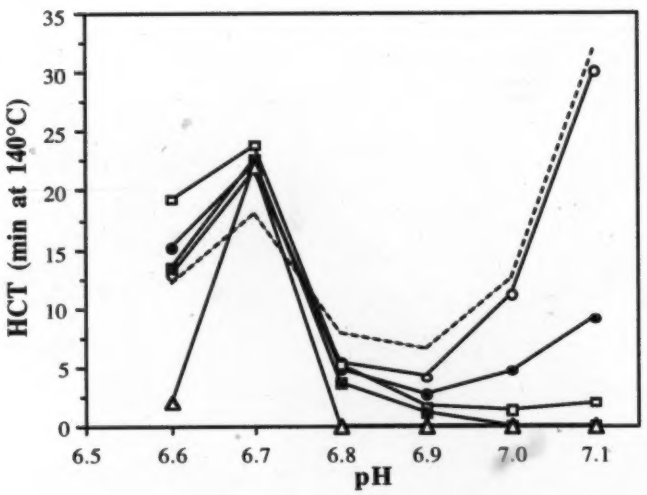

Figure VIII-2. Heat coagulation time (HCT) of pH-adjusted, blends of skim milk and α-fraction solution. The ratio of skim milk to α-fraction solution was 100/0 (- - -), 95/5 (○), 90/10 (●), 70/30 (□), 50/50 (■) or 40/60 (△).

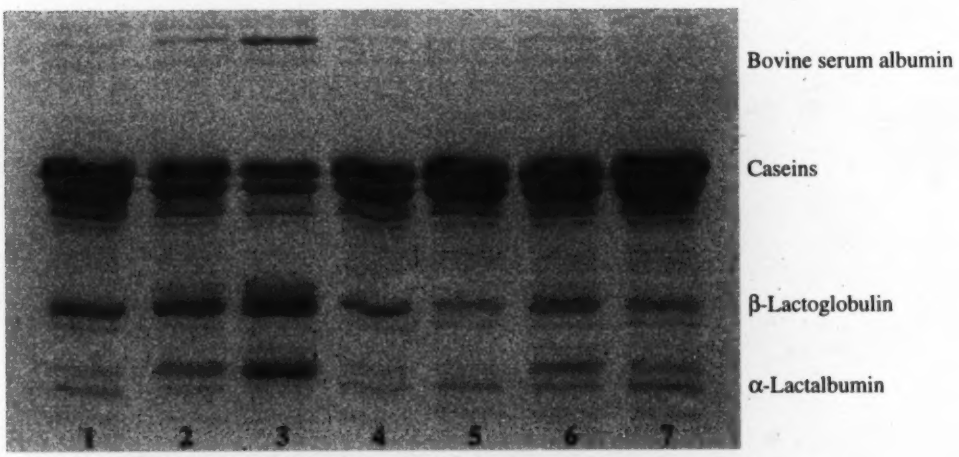

Figure VIII-3. Electrophoretic profile of ultracentrifugal pellets obtained from blends of skim milk and α-fraction solution, which had been pH adjusted, heated at 120°C for 10 min, and cooled to 20°C. At pH 6.6, the ratio of skim milk to α-fraction solution was (1) 100/0, (2) 60/40 or (3) 40/60; at pH 6.8 it was (4)100/0 or (5) 50/50; at pH 7.1 it was (6) 100/0 or (7) 50/50.

in the blends enhanced their thermal association with casein, especially for α-la, the most plentiful protein. The whey proteins must have been complexed to the casein in order to be sedimented at 100 000 x g for 1 h, because, unlike casein, their sedimentation coefficients would be too low to permit exclusive sedimentation, as was confirmed; ultracentrifugation of unheated or heated (120°C/5 min) solutions of α-F, with 1.7% (w/w) protein and pH values in the range 6.6 - 7.1, caused only minute quantities of material to be sedimented. Therefore, heating of skim milk caused α-la to co-aggregate with the caseins, as opposed to a much more limited tendency for self-aggregation in the absence of other proteins, which can be inferred from its high heat stability in the SMP (Figure VIII-1). Law *et al.* (1994) showed also a complexation reaction between casein and α-la; heating milk at natural pH and 80 - 140°C for 5 min caused extensive complexation of α-la, β-lg and bovine serum albumin with casein, allowing these proteins to co-precipitate with casein when the cooled milk was adjusted to pH 4.6. The work of Calvo *et al.* (1993) may indicate that the natural presence of β-lg in skim milk is necessary for α-la to react with casein micelles. However, the heating conditions used in the present study (140°C) were much more severe than that used by the above authors (90°C), obtruding a direct comparison between the two studies.

At initial pH 6.6 or 6.7, the high heat stability of the blends of skim milk and α-FS may have been due to greater thermal complexation of α-la onto casein micelles, increasing micellar charge and hydration and thus heat stability, as postulated by Fox and Hearn (1978). The presence of a small amount of β-lg in the α-F may have caused the decline in heat stability at pH 6.6 and a 40/60 ratio, consistent with the increased concentration of β-lg in the UC pellet of the heated mixture (Figure VIII-3, column 3). This seems likely because at 140°C and pH 6.6 the α-FS had a HCT of ~ 11 min, while the β-FS coagulated almost instantly (Figure VIII-1). Furthermore, skim milk with low levels of added β-FS was very unstable to heating due to independent coagulation of β-lg and/or β-lg complexed to casein (see below).

At pH 6.8 or 7.1, UC pellets from heated blends of skim milk and α-FS did not contain significant quantities of any whey protein (Figure VIII-3, columns 4 - 7), indicating that the added whey protein component was soluble during heating and the low heat stabilities of the blends were caused by coagulation of casein alone; yet the casein became more heat labile in the presence of extra whey proteins. The low heat stability of type A milk at pH 6.9 may be due to the formation of soluble whey protein/κ-casein complexes

and heat labile κ-casein-depleted micelles (Singh and Fox, 1985, 1986, 1987a, b); possibly, in the skim milk/α-FS blends, the rate of this destabilizing interaction would be increased because of a greater concentration of α-la and/or the contaminant β-lg.

Heat stability of blends of skim milk and β-fraction solution

Skim milk in combination with β-FS had a substantially different heat stability pattern, compared to skim milk mixed with α-FS; this was expected in view of the starkly different thermal properties of the α-FS and β-FS (Figure VIII-1). In most cases, blends of skim milk and β-FS showed a greatly reduced heat stability, compared to normal skim milk (Figure VIII-4). For all combinations of skim milk and β-FS, the zone of minimal heat stability deepened and was extended to more alkaline pH values. At initial pH 6.7, low levels of added β-FS caused a modest increase (~ 5 min) in heat stability, but when the ratio of β-FS to skim milk was > 10/90, heat stability declined markedly. At a 70/30 ratio of skim milk to β-FS and at pH 6.8, the HCT was ~ 15 min, compared to a HCT value of ~ 7 min for skim milk at pH 6.8; it appeared that the entire HCT-pH curve was shifted to more alkaline pH values. At all pH values, 50/50 mixtures were extremely unstable; maximum heat stability of only about 5 min occurred at pH 6.8.

Gel electrophoresis of the UC pellets of heated (120°C for 5 min) 50/50 blends revealed that large quantities of β-lg, as well as casein, were present (Figure VIII-5), suggesting that the heat sensitivity of the mixtures was caused by coagulation of β-lg alone and/or of β-lg which had heat-aggregated with casein. Unlike α-la, β-lg was unstable in SMP at 120 and 140°C. Accordingly, the low heat stability at 140°C of the skim milk/β-FS blends could have resulted from exclusive coagulation of β-lg or β-lg/casein micelle complexes or both, all of which would be sedimentable by ultracentrifugation. Compared to mixtures of skim milk with α-FS at pH 6.6 (Figure VIII-2, columns 1 - 3), skim milk with β-FS showed much greater quantities of β-lg in the UC pellets, attesting that denatured β-lg was much more prone to intermolecular interaction with itself and/or casein than was denatured α-la. This conforms with Mottar *et al.* (1989), who reported that a more severe heating of milk was required to induce α-la association onto casein micelles, as compared to β-lg. The rapid thermal coagulation of β-lg in SMP (Figure VIII-1) indicates also a high propensity of denatured β-lg to undergo intermolecular association.

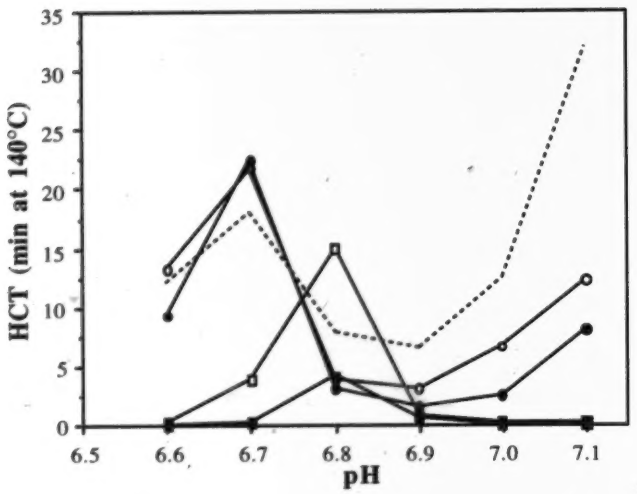

Figure VIII-4. Heat coagulation time (HCT) of pH-adjusted, blends of skim milk and β-fraction solution. The ratio of skim milk to β-fraction solution was 100/0 (- - -), 95/5 (○), 90/10 (●), 70/30 (□) or 60/40 (■).

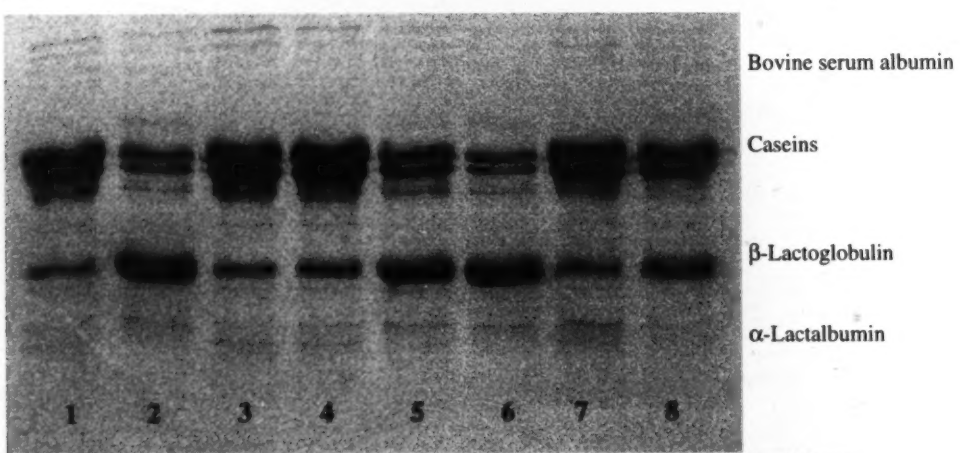

Figure VIII-5. Electrophoretic profile of ultracentrifugal pellets obtained from blends of skim milk and β-fraction solution, which had been pH adjusted, heated at 120°C for 10 min, and cooled to 20°C. At pH 6.7, the ratio of skim milk to β-fraction solution was (1) 100/0 or (2) 50/50; at pH 6.8 it was (3)100/0, (4) 90/10, (5) 70/30 or (6) 50/50; at pH 7.1 it was (7) 100/0 or (8) 50/50.

Because the β-FS alone was heat sensitive, it appears that at pH 6.8 and with a 70/30 ratio, the heat stable entity must have been a β-lg/casein micelle complex, which the SDS-PAGE results also indicate (Figure VIII-5, column 5), and that β-lg did not coagulate exclusively. The result indicates that a shift of the heat stability/pH curve to more alkaline pH values occurred. When normal skim milk is heated at pH 6.7, β-lg heat-precipitates onto the surface of casein micelles to increase their heat stability (Singh and Fox, 1985, 1986, 1987a, b); the peak heat stability of the 70/30 mixture at pH 6.8, indicates that in the presence of extra β-lg, a favourable degree of interaction between β-lg and casein occurred at a higher pH value. The slightly improved heat stability, at pH 6.7, of 95/5 or 90/10 combinations, may have been due also to thermal complexation between β-lg and casein micelles. At > pH 6.9, all combinations of skim milk and β-FS had low heat stabilities, possibly because of a more rapid formation of soluble κ-casein/β-lg complexes and hence heat labile micelles and/or the sole aggregation of β-lg.

Patocka *et al.* (1993) showed that combinations of skim milk and acid whey UF retentate, at pH 6.5 - 6.7, coagulated at 90°C; the high calcium content of the UF retentate promoted formation of casein/whey protein co-precipitates. The same authors found that mixtures of skim milk and sweet or UC whey UF retentate were heat stable at 90°C and pH 6.5 - 6.7, attributed to lower levels of calcium in the UF retentate. The blends of skim milk and β-FS, used in the current study, would be expected to be similar in composition to blends of skim milk and sweet or UC whey used by Patocka *et al.* (1993). The results here imply that without elevated calcium levels, the presence of extra β-lg was also detrimental to the heat stability of skim milk, the effect occurring over a range of pH values, albeit at a higher temperature (140°C).

Heat stability of blends of skim milk and whey protein concentrate solution

Combinations of skim milk and WPCS had HCT-pH profiles (Figure VIII-6) broadly similar to skim milk/β-FS blends, indicating that heat stability was strongly influenced by an increased concentration of β-lg, originating from the WPC.

In most cases, blends of WPCS and skim milk were much less heat stable than skim milk, a conspicuous exception being a pronounced increase in the HCT (to a value of ~ 22 min) at pH 6.8, when the ratio of skim milk to WPCS was 70/30. The HCT/pH profile was shifted to more alkaline pH values, as also occurred with a 70/30 ratio of skim

milk to β-FS. Gel electrophoresis of UC pellets obtained from heated blends of skim milk and WPCS (results not shown) led to a very similar pattern to that observed for the combinations of skim milk and β-FS (Figure VIII-5); this implies that the heat stability pattern was again due primarily to β-lg and the heat instability may have been caused by aggregation of β-lg alone and/or β-lg in association with casein. Similarly, the high thermal stability of a 70/30 blend, at pH 6.8, appeared to be due to the formation of a complex between β-lg and casein.

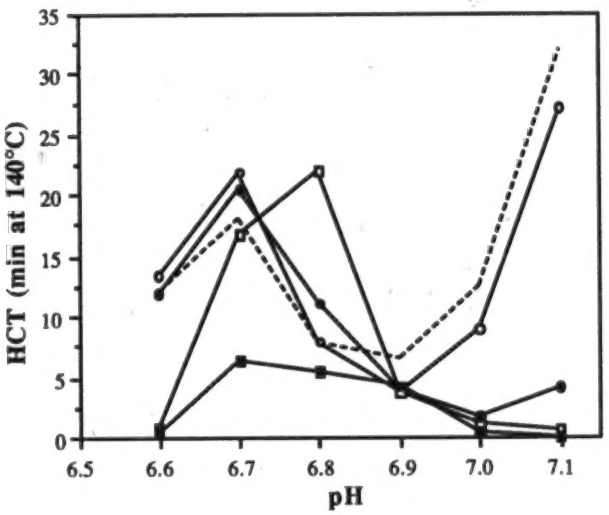

Figure VIII-6. Heat coagulation time (HCT) of pH-adjusted, blends of skim milk and whey protein concentrate solution. The ratio of skim milk to whey protein concentrate solution was 100/0 (- - -), 95/5 (○), 90/10 (●), 70/30 (□) or 60/40 (■).

Studies have shown that whey protein products rich in β-lg have similar thermal properties to more purified β-lg preparations (de Wit, 1981; Hines and Foegeding, 1993), so it should not be surprising that the heat stability and gel electrophoretic patterns of skim milk combined with either β-FS or WPCS were similar. However, the actual HCT values of skim milk/WPC solution combinations were higher, implying an effect of other proteins and/or the non-protein constituents in the WPC, the nature of which was not established.

Conclusions

The thermal behaviour of whey protein preparations was dominated by their most abundant protein constituent; α-la in the α-F; β-lg in the β-F or the WPC. Confirming the well known differences in the heat stabilities of α-la and β-lg, the α-F was resistant to coagulation in SMP at temperatures up to 140°C, while the β-FS quickly formed visible protein clots at 140°C. This was probably due to a greater degree of thermal unfolding for β-lg as compared to α-la, implying that the overall heat stability of α-la, in terms of extent of conformational changes and aggregation, is greater than that of β-lg.

The heat stabilities of blends of skim milk and whey protein solutions were dependent on the type of whey protein preparation used and the initial pH. At the unaltered pH (6.7) of the mixtures, α-la had a beneficial effect on heat stability up to at least a 40/60 ratio of skim milk to α-FS, whereas β-lg caused a small increase in heat stability at < 90/10 ratio but was extremely deleterious at higher levels of addition. In general, at pH values > 6.7, extra α-la or especially β-lg caused the heat stability to decline; exceptions were the high heat stabilities, at pH 6.8, of 70/30 combinations of skim milk and β-FS or WPCS.

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- Chapter IX -

Freezing point depression of milk as an indicator of the effects of pH, heat treatment and ionic strength on the partitioning of milk salts*

Introduction

As described in section 2.3 of Chapter II, the milk salt system is dynamic, in the sense that the distribution of total milk salt between the colloidal and soluble states depends on a number of factors, including pH, temperature, the presence of additives and concentration of milk by evaporation or ultrafiltration (UF). Some of the methods used to quantify the effects of these factors on the milk salt system are tedious to employ and may distort the salt equilibria. These include the use of UF, dialysis or ultracentrifugation of milk, to fractionate the salts into their colloidal and soluble phases and quantifying the types of minerals in each phase. As discussed by Holt (1985), the existence of a 'Donnan potential' and excluded volume effects contribute to uncertainties in the use of these techniques. The use of ion selective electrodes to measure the activity of specific ions, such as calcium, sodium, chloride or potassium, has the advantage that ion activity can be measured in the milk itself, without the need to separate soluble from colloidal salts but the reliability of these electrodes needs to be carefully checked if the pH or temperature of the milk are altered.

In section 2.4.4 of Chapter II, it was pointed out that measurement of freezing point (FP) of milk might allow for the sensitive and rapid determination of changes in milk salt partitioning and that the changes could be quantified in milk itself, without the need for separation procedures. The use of FP measurements to assess changes in the milk salt system has already been seen to a limited extent in Chapters IV and VII. In Chapter IV, it was observed that the addition of skim milk UF permeate or sweet whey UF permeate to milk caused the FP to increase, whereas addition of acid whey UF permeate had the opposite effect. In Chapter VII, FP measurements showed that direct or indirect ultra-high temperature heating did not lead to irreversible changes in the milk salt partitioning.

In this Chapter, a more rigorous evaluation of the use of FP measurements to monitor changes in milk salt equlibria is presented. The influences of (1) acidification (2) alkalization or (3) acidification followed by alkalization, on the FP of unheated or

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* A version of this Chapter was submitted to *J. Dairy Res.* for publication.

heated (80 - 140°C) skim milk were determined, in an attempt to evaluate the impact of these heat treatments on the distribution of milk salts between the soluble and colloidal phases. The FP of milk which had been heat-coagulated was also measured in an attempt to elucidate the influence of milk salts on the heat coagulation process. Additionally, the effects of added salts (NaCl, LiCl, $CaCl_2$ or Na_2SO_4) on the FP of milk were determined to ascertain if the added salts would bind to protein or be dissolved in the aqueous phase of milk and how this would relate to the protein salting-out phenomenon.

Materials and methods

Adjustment of pH

The pH of unheated or heated skim milk was adjusted by the addition of 6.7 N HCl or 5.0 N NaOH. Three types of experiments involving pH adjustment were carried out: (1) Acidification of milk to pH values in the range ~ 6.7 - 4.8, where pH ~ 6.7 was the unaltered pH of skim milk; (2) Acidification of milk to pH 4.8, followed by immediate neutralization to obtain milk samples with pH values in the range ~ pH 4.8 - 10.0; (3) Alkalization of milk to pH values in the range ~ 6.7 - 10.0. All pH adjustments were carried out at 4°C, and involved the progressive addition of 5 μL quantities of acid or alkali to 100 mL of milk that was being rapidly stirred. Following pH adjustment, the unheated or heated milk samples were stored at 4 °C for 20 h, equilibrated to 20 °C for 4 h, after which the pH and FP were measured.

Heat treatment

Aliquots (~ 30 g) of skim milk, at unaltered pH (~ 6.7) were placed in a glass tubes (internal diameter, 14 mm), the tubes sealed and placed in an oil bath, and the milk samples heated quiescently at 80 °C for 3 h or 110 - 120°C for 20 min. After heating, the milk samples were cooled quickly to 4 °C and stored at this temperature for 20 h, prior to adjustment of pH and measurement of FP. In some experiments, the pH of milk was adjusted to values in the range 6.35 - 7.1, and the milk heated at 130°C to its coagulation point, determined as the heat coagulation time (HCT) at 130°C, the same method used as in Chapter III. After heat coagulation, the milk was cooled quickly to 4°C, stored at 4°C for 20 h and then equilibrated at 20°C for 4 h; the pH and FP of the heat-coagulated milk were then measured.

In this work, pasteurized skim milk which had not been subjected to any experimental heat treatment is designated as 'unheated milk'. Though this is technically incorrect, the term is convenient to use and it may be justified on the basis that the pasteurization heat treatment (72°C/15 s) is very mild compared to the experimental heat treatments used.

Addition of salts

Quantities of NaCl, LiCl, $CaCl_2$ or Na_2SO_4 (Sigma Chemical Co., St. Louis, MO), were dissolved directly in pasteurized skim milk at 20°C, to obtain calculated added solute concentrations in the range 0 - 1000 mmolal (mmoles solute L^{-1} solvent). As a control, the same salts were dissolved in double-distilled, deionized water at the same concentration range; if an added salt had bound to milk protein, then its capacity to lower the FP of milk would have been less than that in water. The samples were stored at 4°C for 20 h, then equilibrated at 20°C for 4 h, after which the FP's were measured.

Measurement of freezing point

The FP of each milk sample was measured using a Model 3300 Osmometer (Advanced Instruments Inc., Norwood, MA), which required 50 µL of sample and measured the FP according to the principle described in section 2.4.2 of Chapter II. The instrument gave readings in molality, which were converted to FP values by multiplying by a factor of - 1.86, using the formula shown in section 2.4.1 of Chapter II. Individual experiments, involving heat treatment or adjustment of pH or ionic strength, were carried out on three separate occasions, and the FP values determined at least in duplicate.

Results and discussion

Influence of pH adjustment on the freezing point of unheated or heated skim milk

The effects of acidification, alkalization or acidification followed by alkalization on the FP of unheated or heated skim milk are shown in Figure IX-1, from which the following trends can be seen: (1) Addition of HCl to unheated or heated skim milk caused the FP to decrease gradually and the FP/pH profile was not altered by even the most severe heat treatment (120°C/20 min); (2) When unheated or heated milk was acidified to pH 4.8 and then neutralized to pH 10, the FP remained low and relatively constant and heating did not

alter this trend; (3) A small decline in pH occurred when milk was heated, which was accompanied by a reduced FP, the effect becoming more pronounced with increasing severity of heating; this pH shift had no effect on the FP/pH profile upon acidification or acidification followed by alkalization; (4) Alkalization of milk, up to ~ pH 10, had little effect on the FP, except for the most severely heated milk sample (130°C/20 min) which showed a consistently lower FP than the other milk samples.

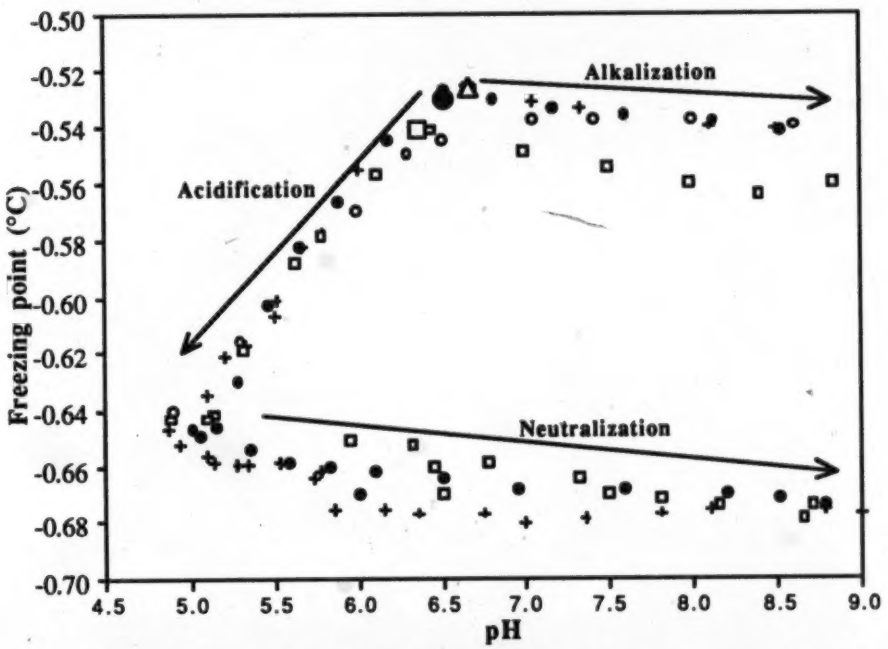


Figure IX-1. Influence of acidification, acidification followed by neutralization, or alkalization on the freezing point of skim milk, which was unheated (+), heated at 80°C/3 h (o), 110°C/20 min (●) or 120°C/20 min (□). Acidification or alkalization commenced at the natural pH of the milk as indicated by large symbols; Δ, unheated milk or milk heated at 80°C/3 h; ●, milk heated at 110°C/20 min; □, milk heated at 120°C/20 min.

The reduction in FP caused by acidification of milk was almost certainly due to the dissolution of CCP alone, leading to an increase in the concentration of soluble salts in the serum phase of milk. It might also be expected that the addition of HCl would increase the molality of milk and hence contribute also to a lower FP, because addition of HCl would cause the concentrations of soluble H^+ ions $[H^+]$ and soluble Cl^- ions $[Cl^-]$ to increase. However, even at pH 4.8, the $[H^+] + [Cl^-]$ were calculated to be extremely low, making a minute contribution to the FP, as shown below:

$pH = - \log_{10} aH^+ \approx - \log_{10} [H^+]$

$\Rightarrow$ at pH 4.8, $[H^+] \approx \text{antilog}_{10} (- 4.8) = 1.58 \times 10^{-5}$ Molar

$\Rightarrow [H^+] + [Cl^-] = 3.17 \times 10^{-5}$ Molar $\approx 3.17 \times 10^{-5}$ Molal

$\Rightarrow$ FP depression due to acidity = $1.86 \times 3.17 \times 10^{-5} = 5.89 \times 10^{-5}$ °C

This was confirmed experimentally; when 10 µL of 6.7 N HCl was added to 10 mL of water, it was calculated (because the pH of dilute aqueous solutions cannot be measured satisfactorily) that the pH would be ~ 3, and the measured FP was close to 0 °C. Therefore, it can be concluded that the reduced FP upon acidification of skim milk was due almost entirely to the dissolution of CCP.

Likewise, it can be shown that the addition of NaOH to milk has an extremely low impact on the FP, even for the greatest addition of alkali to pH 10:

$pOH = - \log_{10} aOH^- \approx -\log_{10} [OH^-] = 14 - pH \Rightarrow$ at pH 10, pOH = 4.0

$\Rightarrow$ at pOH 4.0, $[OH^-] \approx \text{antilog}_{10} (- 4.0) = 1.0 \times 10^{-4}$ Molar

$\Rightarrow [OH^-] + [Na^+] = 2.0 \times 10^{-4}$ Molar $\approx 2.0 \times 10^{-4}$ Molal

$\Rightarrow$ FP depression due to alkalinity = $1.86 \times 2.0 \times 10^{-4} = 3.72 \times 10^{-4}$ °C

As seen in Figure IX-1, when skim milk was heated at 110 or 120°C for 20 min, a small but irreversible reduction of pH took place, which was accompanied by a slightly lower FP: unheated milk had a pH of 6.7 and a FP of - 0.522°C; milk heated at 80°C/3 h displayed a pH of 6.67 and FP of - 0.522°C; milk heated at 110°C/10 min had a pH of 6.55 and FP of - 0.525°C; milk heated at 120°C/20 min showed a pH of 6.44 and FP of - 0.542°C. The decline in the pH of milk upon heating at high temperature has been attributed to three effects (see section 4.2, Chapter II): (1) The degradation of lactose into organic acids; (2) Conversion of soluble primary calcium phosphate into insoluble secondary and tertiary calcium phosphates with release of H^+ ions; (3) Dephosphorylation of the caseins accompanied by the reaction of the released phosphate groups with calcium to generate tertiary calcium phosphate and H^+ ions. The degradation of lactose into organic acids would almost certainly be an irreversible process and probably contributed to most of the irreversible pH decline. The formation of secondary and tertiary calcium phosphates with release of H^+ ions is likely to be a reversible process. Geerts *et al.* (1983) found that when milk was heated at 115°C for ~ 1 - 11 min and then cooled to 20°C, the aCa^{2+} increased logarithmically with time, indicating that heating increased the concentration of

soluble salts but the change was rapidly reversible upon cooling. The dephosphorylation of casein upon heating of milk is reported to be irreversible and therefore may also have contributed to the irreversible decline of pH, but to what extent is unknown; data on the extent of heat-induced dephosphorylation of the caseins in milk are incomplete and rather equivocal; values of ~ 12% for milk heated at 120°C for 90 min, ~ 18% for milk heated at 120°C for 30 min or ~ 65% dephosphorylation for milk heated at 130°C for 30 min, have been reported (Dalgleish *et al.*, 1987; Singh, 1995). Moreover, Dalgleish *et al.* (1987) showed that upon dephosphorylation of the caseins, the released phosphate groups remained within the casein micelle, which casts doubt as to whether dephosphorylation does contribute to the heat-induced pH decline of milk.

The reduced FP, which accompanied the irreversible heat-induced decline in the pH of milk heated at 110 - 120°C/20 min, was probably caused by a greater dissolution of CCP. However, the FP/pH profile corresponding to acidification was not altered by heat treatment; for milk heated at 120°C for 20 min, acidification commenced at a lower natural pH (6.44) and a lower FP (- 0.542°C), but these values did not deviate from the shape of the FP/pH curve. In the case of less severely heated milk, no change in FP occurred, probably because the magnitude of pH drop (0.02 or 0.15 pH units for milk heated at 80°C for 3 h or 110°C for 20 min, respectively) was too small to cause noticeable dissolution of the CCP.

The works of Geerts *et al.* (1983), Pouliot *et al.* (1989), Lucey *et al.* (1993a, b, 1996), Law (1996) and Singh *et al.* (1996), as discussed in section 2.3.2 of Chapter II, indicate that when milk is heated, a shift from soluble to colloidal salts occurs, but upon subsequent cooling the salt system reverts to its native state. Moreover, as argued by Holt (1995), it appears that the structure of indigenous CCP is not altered by heating. The current results show that heating followed by cooling had no effect on the FP/pH profiles of acidified milk or acidified and subsequently neutralized milk. This upholds the view that the partitioning of milk salts or the structure of indigenous CCP is not irreversibly altered by heating, even when heating is as severe as 120°C for 30 min.

Addition of NaOH to unheated or heated skim milk, at its natural pH, caused almost no change in the FP. It appeared that alkalization had no effect on the partitioning of milk salts, which can also be concluded from the work of Lucey *et al.* (1993a), who reported little change in the buffering index of milk over the pH range ~ 6.7 - 8.0, indicating that

changes in the structure of indigenous CCP were unlikely to occur. Compared to unheated or less severely heated milk, samples heated at 120°C for 20 min, showed FP values that were consistently higher upon alkalization. This was probably because alkalization commenced at pH 6.44 (the natural pH of milk heated at 120°C/20 min), where limited dissolution of CCP had taken place. The effect confirmed the inability of dissociated CCP to reassociate with the casein micelles, which was also observed, albeit much more profoundly, when milk was acidified to pH 4.8.

Influence of heat-induced coagulation on the freezing point of milk

The influence of initial pH on the heat stability of milk, determined as the HCT at 130°C, and the extent of pH shift caused by heating milk to its coagulation point are shown in Figure IX-2. The HCT/pH curve indicates a type A milk, with a pronounced minimum near pH 6.8; the thermal behaviour of type A milk was discussed in detail in section 4 of Chapter II.

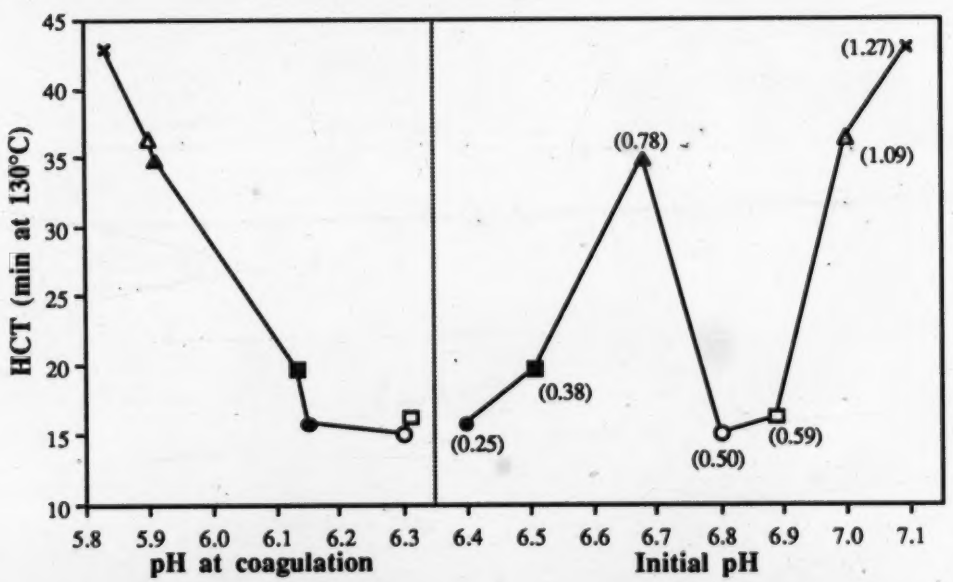


Figure IX-2. Heat coagulation time (HCT) of skim milk as a function of initial pH and the pH at coagulation point. Alike symbols correlate the initial pH and the pH at coagulation time for the same milk samples. Numbers in brackets indicate the extent of pH drop caused by heating milk at each initial pH value.

Of interest here, was the decline in pH which occurred when milk samples were heated to their coagulation points. Excluding the zone of the minimum (pH 6.8 - 6.9), the higher the initial pH of milk, the greater was heat stability, the greater was the pH shift upon heating and the lower was pH at the coagulation point. In the zone of the minimum, a much smaller pH shift occurred upon heating of milk to its coagulation point. The results indicate that the mechanism of heat-induced coagulation must have been different for each initial pH value and that heat stability was not merely a type of acid coagulation. Otherwise, one would expect all milk samples to coagulate at the same pH value, and heat stability to be determined solely by factors which affect the rate of pH decline; the complicated relationship between heat-induced pH shift and heat stability was discussed by van Boekel *et al.* (1989).

In the region of minimum (initial pH 6.8 - 6.9), it may be that the formation of κ-casein-depleted micelles, would necessitate a much smaller pH drop (0.50 - 0.59) for heat coagulation to occur, because depletion of κ-casein is accompanied by a loss of steric and electrostatic intermicellar repulsive forces. Alternatively, it may be that a pH shift has little bearing on the heat coagulation process in the zone of minimal heat stability, and that as soon as κ-casein depleted micelles form they coagulate, giving little time for the reactions (degradation of lactose, precipitation of salts, dephosphorylation) responsible for the pH decline to occur. The large pH shift (1.27) which accompanied coagulation at initial pH 7.1, suggests that κ-casein-depleted micelles may have formed initially, but then were altered to increase their heat stability. It was proposed by van Boekel *et al.* (1989) that when the initial pH of milk was > 6.9, soluble complexes between whey proteins and κ-casein formed during the initial stages of heating, but subsequently associated onto the surface of the micelles, increasing micellar heat stability; if this is true is could account for the necessity of a large decline in pH for coagulation to occur. At pH 6.4 - 6.7, the heat stability of casein micelles, with surface complexes between whey proteins and κ-casein is relevant. If it is assumed that a lower pH leads to a reduced surface charge on the casein micelles, then, as evident from Figure IX-2, one has to conclude that the lower the initial surface charge of the casein micelle, the higher was its surface charge at the heat coagulation point. However, this is probably not the case as there is no direct relationship between pH and micellar ζ-potential and in fact such a relationship is poorly understood (Schmidt and Poll, 1986; Anema and Klostermeyer, 1996). Additionally, it must be

emphasized that the pH values reported here are not truly representative of the pH at coagulation, as pH could not be measured at 130°C.

The FP data for unheated milk or milk which had been heated to its coagulation point, as affected by pH, are shown in Figure IX-3; for unheated milk, the pH was adjusted by the addition of small amounts of HCl, while for heat-coagulated milk, the pH at the heat coagulation point was not further adjusted. In both cases, the FP decreased as the pH was reduced, probably due to increased solubilization of CCP. However, heat-coagulated milk had slightly higher FP values than unheated milk, at similar pH values, which may indicate that an irreversible precipitation of soluble salts occurred when milk was heated to its coagulation point.

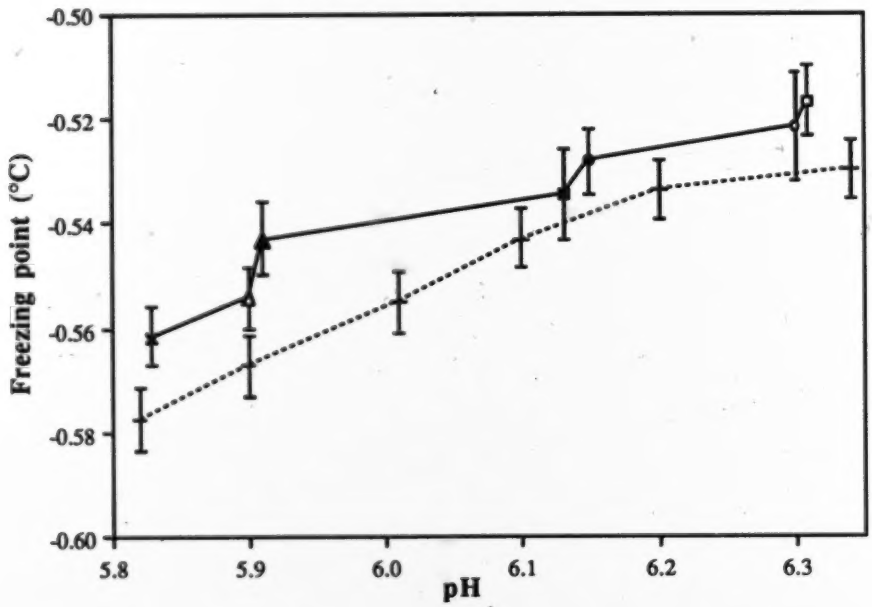


Figure IX-3. Freezing point of unheated skim milk (--+--) as a function of pH, or skim milk samples heated to their coagulation points, causing the pH to decline to the values indicated (the same pH values as in Figure IX-2, as indicated by identical symbols). Error bars represent the standard deviation for the FP values of each data point.

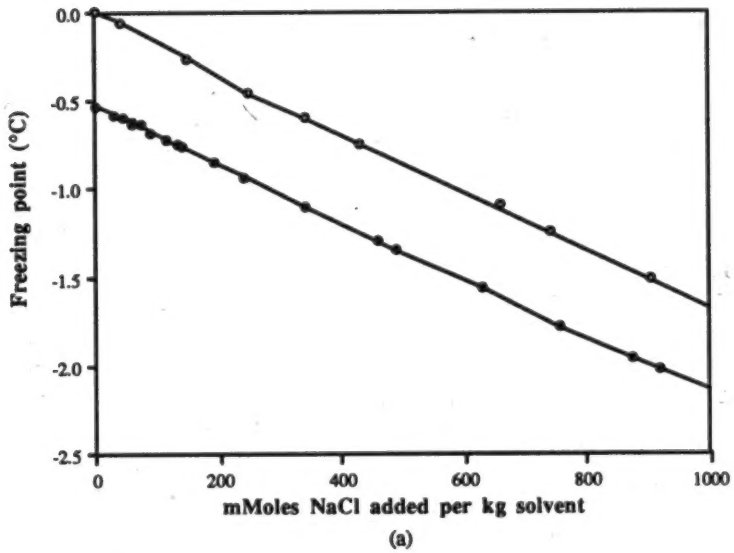
As noted previously, less severe heating of milk, which did not lead to clotting, apparently had no irreversible effect on the milk salt partitioning, even though the heating regimes used were still relatively harsh (up to 120°C for 20 min). In the case of heat-coagulated milk samples, the higher FP values could have been caused by the conversion

of primary calcium phosphate into less soluble tertiary calcium phosphate. As discussed above, this change is believed to be responsible for part of the heat-induced decline of milk pH. Therefore, it is possible that part of the irreversible pH decline of heat-coagulated milk was caused by a reduced concentration of soluble salts, as well as by the degradation of lactose. It is notable that the extent of pH decline in milk heated to its coagulation point was much greater than that for less severely heat milk; for example, when milk at pH 6.7 was heated at 120°C/20 min the pH declined to 6.44 (ΔpH = 0.26), but when heated to its coagulation point the pH was reduced to 5.92 (ΔpH = 0.78).

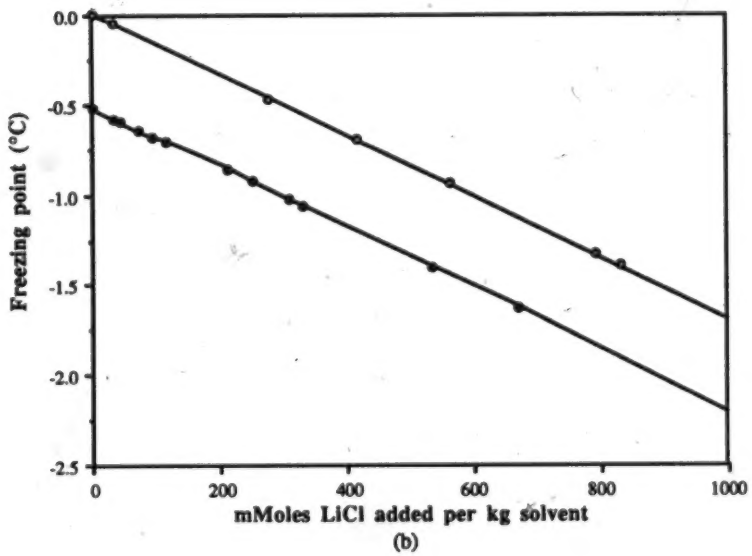
Influence of added salts on the freezing point of milk

The effects of added NaCl, LiCl, $CaCl_2$ or Na_2SO_4 on the FP of milk or water are shown in Figure IX-4. In all cases there was a linear relationship between FP and the moles of salt added per kg of solvent, whether the salts were added to milk or to water. In the case of all four salt species, there was no difference between the slopes of the plot for water compared to milk; the two lines were parallel by a difference of 0.522°C, which corresponds to the FP of normal milk. This indicates that the addition of salts to milk did not alter the 'background' salt equilibria of milk and the added salts did not bind to protein. Instead the added salts were dissolved in the aqueous phase of milk, where they caused the FP to decrease at exactly the same rate as if they had been added to water.

Individual caseins and whole casein are capable of binding to metal ions, especially Ca^{2+} (Rollema, 1992), but such effects have been characterized in model systems at low ionic strength, rather than in milk. The inability of casein in milk to bind to any of the added salts indicates that all of the binding sites for salts were already occupied, at the relatively high ionic strength of milk. The major binding sites for salts in the caseins are the phosphoseryl residues, which are already bound to the indigenous CCP. Although the glycomacropeptide portion of κ-casein is negatively charged and exposed to the solvent, the present results suggest that it did not bind to any of the cations generated by the added salts. In fact, the loss of glycomacropeptide from casein, by the action of chymosin, causes aggregation of casein micelles and such aggregation is assisted by the presence of Ca^{2+}, suggesting that the binding of calcium to casein micelles is increased when the glycomacropeptide is lost.

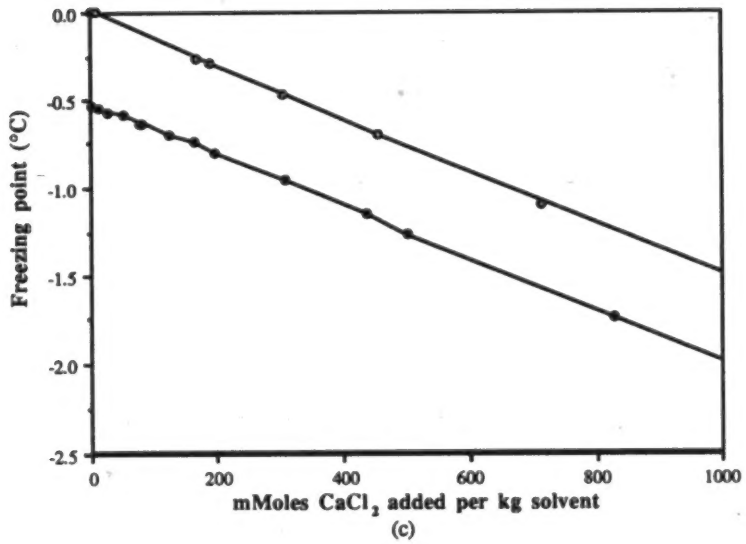


(a)

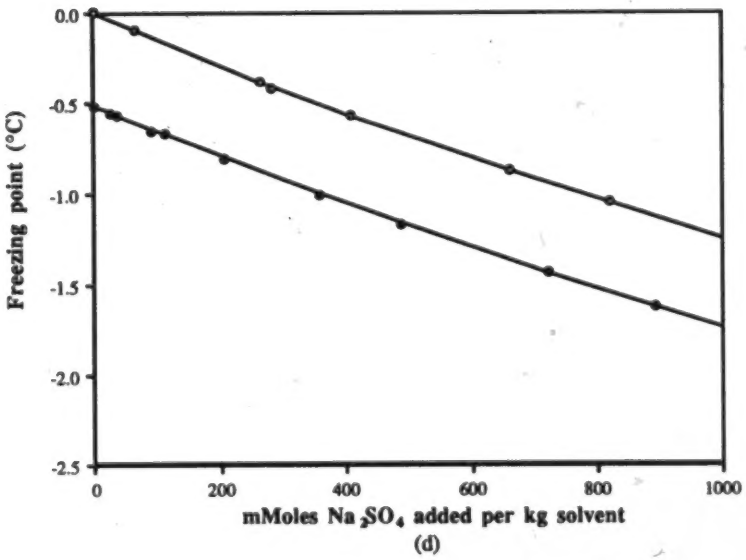


(b)

Figure IX-4. Influence of (a) added NaCl or (b) added LiCl on the freezing point of water (O) or skim milk (●).



(c)



(d)

Figure IX-4 (-ctd-). Influence of (c) added $CaCl_2$ or (d) added Na_2SO_4 on the freezing point of water (O) or skim milk (●).

In general, for many proteins an increase in solubility (salting-in) occurs when ionic strength is increased from zero, but at higher ionic strength solubility is reduced (salting-out). The results here suggest that in milk, the caseins are already salted-in and the addition of extra salts causes them to be ultimately salted-out; the formation of visible clots was observed upon the addition of ~ 0.8 moles of Na_2SO_4, ~ 1.7 moles of $CaCl_2$, ~ 4.3 moles of NaCl or 7.6 moles of LiCl, to 1 kg of skim milk, equivalent to ~ 11.5% Na_2SO_4, ~19% $CaCl_2$, ~ 25% NaCl or ~ 31% LiCl.

Using a variety of salt types, Arakawa and Timasheff (1984) showed that salting-in of bovine serum albumin or lysozyme may have been caused by the binding of salt ions to proteins to increase protein solubility; this was accompanied by a reduction in the partial specific volume of the protein, possibly because protein salting-in would lead to tighter protein folding. At higher high ionic strengths, it was found that, for most salt types, the partial specific volumes of the proteins increased, indicating a greater ordering of water molecules near the protein surface or 'preferential hydration' of the protein. It was proposed that 'preferential hydration' was a consequence of added salt ions being excluded from the vicinity of the protein surface. The preferential hydration of protein might be expected to provide a driving force for increased protein association, because the presence of a larger hydration shell would reduce the entropy of solvent in the vicinity of the protein, the effect of which could be counteracted by reducing the surface area of protein exposed to solvent via intermolecular protein association. Arakawa and Timasheff (1984) showed that among various salt species, Na_2SO_4 induced the greatest preferential hydration of protein and displayed the greatest efficacy for salting-out, the latter observation being consistent with the present results. Obviously, great caution would be required in extending the somewhat speculative postulate of Arakawa and Timasheff (1984) to the case of salting-out of milk proteins, especially for the case of high molecular weight casein micelles. However, it can be said that FP measurements indicated that the addition of salts to milk did not result in their binding to milk proteins.

The ability of co-solutes to induce preferential hydration of proteins is also the mode of action of cryo- or thermoprotectants, such as salts and various simple sugars (Timasheff and Arakawa, 1988; Arakawa *et al.*, 1990; Carpenter *et al.*, 1990); protein denaturation is hindered because the hydration shell of a denatured protein would be much greater in the presence of a co-solute, creating an entropically unfavourable situation.

Conclusions

The FP of milk was sensitive to the concentration of soluble salts, which in turn was affected by environmental conditions, such as temperature and pH. Therefore, measurement of FP provided a convenient and rapid method to monitor changes in milk salt equilibria. However, FP measurement could only be used to assess changes in total soluble milk salt content, rather than that of specific soluble ionic species. Furthermore, FP measurements were made at the same temperature (by supercooling milk to - 3°C); thus, only the irreversible effects of heating on milk salt equilibria could be detected. Despite these difficulties, FP measurement showed conclusively that the acid dissolution of CCP was a totally irreversible process, as subsequent alkalization did not lead to any increase in FP. In contrast, the well-known shift from soluble to colloidal salt caused by heating of milk appeared to be almost completely reversible upon cooling, unless the milk was heated to its coagulation point, which may have caused the irreversible precipitation of calcium phosphate. Measurement of FP showed convincingly that the addition of salts to skim milk, to concentrations at least as high as 1000 mmolal, did not result in their binding to milk proteins or insolubilization; the FP of milk declined in a linear manner, exactly as predicted for the presence of free, unbound salt ions.

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- Chapter X-

General conclusions and future research needs

The results of Chapters III - IX, though interrelated, may be crystallized into three concise statements, the substance and future implications of which are subsequently elaborated on:

1 Protein standardization

- Skim milk ultrafiltration (UF) permeate (SMP) and rennet casein whey UF permeate (SWP) were found to be befitting materials for the protein 'down' standardization of skim milk or fat-containing milk, in contrast to the unsatisfactory use of acid whey UF permeates, whether derived from acid casein whey (AWP) or from a fermented dairy product (FWP).

The suitability of UF permeates derived from skim milk or sweet whey, in contrast to the unsuitability of acid whey UF permeates for protein 'down' standardization of milk, were demonstrated, on an experimental scale, in terms of the impact of permeate addition on the heat stability and sensory quality of milk. When heat stability was measured as the heat coagulation time, over the initial pH range 6.4 - 7.1, the high heat stability of skim milk became even greater upon standardization with SMP or SWP, while the presence of AWP in milk caused a great reduction in heat stability. When AWP was used, the reduction in heat stability was especially pronounced at the natural pH (~ 6.4 - 6.6) of the protein-standardized milk products. Triangle sensory tests showed that it was very difficult to distinguish between normal skim milk or 2% fat milk and their protein-standardized counterparts, when standardization was carried out with permeates obtained by UF of skim milk or sweet whey, but the use of UF permeate originating from acid whey, especially when the whey was derived from a fermented dairy product, had a negative effect on the sensory quality of milk. Experimental trials showed also the suitability of using skim milk UF retentate for protein 'up' standardization, which caused only a very small reduction in the heat stability of 2% fat milk and did not appreciably alter its sensory quality.

The pilot-scale manufacture of protein-standardized ultra-high temperature (UHT) milk, manufactured by the direct or indirect heating method, provided further confirmation of the suitability of SMP for protein 'down' standardization. During direct or indirect UHT

heat treatment and subsequent storage for up to 12 weeks at 25°C, the protein-standardized UHT milk products exhibited excellent physical stability, equal to, if not greater, than that of normal UHT milk. Detailed compositional analysis revealed that the most notable effect of standardization with SMP on the nutritional quality of milk, apart from a lower protein content, was a slightly reduced concentration of calcium, phosphorus and potassium. In general, the sensory quality of protein-standardized UHT milk, containing SMP, whether made by direct or indirect heating method, was very similar to that of normal UHT milk. In contrast to the use of SMP, attempts to produce UHT milk protein 'down'-standardized with FWP were futile. The UHT milk products with FWP showed slightly better profiles of micronutrients, than those with SMP, but in other respects the use of FWP was unacceptable; the reduced heat stability of milk with FWP necessitated careful adjustment of pH before UHT heating, to avoid protein coagulation in the UHT machine, but a more serious problem was the occurrence of extremely high sedimentation, with phase separation upon storage. Furthermore, the use of FWP for protein 'down' standardization was responsible for the presence of unacceptably strong off-flavours in UHT milk, especially when the milk was processed by indirect UHT heating. Although direct UHT heating caused volatilization of some of the off-flavours, during the vacuum flash removal of infused steam from the milk, the resultant improvement in flavour was inadequate to confer acceptable sensory quality to the UHT milk.

The process of protein standardization of fluid milk, by the use of UF procedures, is in many respects analogous to fat standardization, whereby centrifugal separation divides the milk into fat-enriched and fat-free streams. Similarly, UF of milk produces protein-enriched and protein-free streams, which may be recombined to obtain a range of milk products with different protein contents. This analogy between fat and protein standardization, makes protein standardization by the use of UF an attractive proposition. The results of the present work should provide a further incentive to standardize the protein content of milk by the addition of permeate derived by UF of milk, considering that changes in heat stability are, if anything, beneficial, and changes in sensory or nutritional quality are minimal. At the same time, however, the unsuitability of acid whey UF permeate for protein 'down' standardization was unequivocally demonstrated. As a result of increased manufacture of quarg and other fresh cheese types and acid casein, the production of various types of acid whey and acid whey UF permeate is increasing

(Sienkiewicz and Riedel, 1990), creating a need for effective utilization procedures. Unfortunately, the present results demonstrate that this issue cannot be resolved by use of acid whey UF permeates as protein standardizing agents. In fact, because of its undesirable flavour, it is difficult to envisage how acid whey or acid whey UF permeate, derived from fermented dairy products, could be used as ingredients for any food type. Therefore, it is prudent to rely on and improve the conventional uses of acid whey and acid whey UF permeate, namely, use as an animal feed, fertilizer, extraction of the lactose or fermentation to produce valuable products such as ethanol, methane or single cell protein (Sienkiewicz and Riedel, 1990; Renner and Abd El-Salam, 1991), and to minimize the inane option of treating acid whey permeate as a waste product.

It was demonstrated that UHT milk 'down'-standardized with SMP exhibited excellent stability, in terms of low sedimentation, upon storage, at 4 or 25°C, for up to 3 months. However, the impact of SMP on the age-gelation process of UHT milk, which typically occurs after 6 - 12 months storage, was not established. The general theory of age-gelation envisages that a slow aggregation of casein micelles into a network occurs; casein micelle aggregation occurs due to changes at the micelle surface, which are either enzymatic or physico-chemical in nature (Harwalkar, 1992). Recently, McMahon (1996) proposed an alternative theory of age-gelation, where it was envisaged that during UHT heating β-lg associated onto the surface of the casein micelles, but upon storage of the UHT milk, complexes between β-lg and κ-casein dissociated from the casein micelles and subsequently crosslinked with themselves and with casein micelles to form a network. In the case of milk standardized with SMP, it would be interesting to study the age-gelation process. In view of the fact that casein micelles in such milk exhibited an increased heat stability, the question arises as to whether standardization would also confer to the casein micelles an increased resistance to aggregation and hence age-gelation during the storage of UHT milk. If this would be true, then another incentive for protein standardization with SMP would exist.

The addition of increasing amounts of SMP or SWP to milk for protein 'down' standardization caused the freezing point (FP) to increase progressively; possibly, this effect would reduce the acceptability of protein standardization, as an abnormally high freezing point is normally associated with the presence of added water in milk. Because the addition of SMP or SWP to milk was shown to cause only minor changes in heat stability,

nutritional and sensory quality, equating the presence of SMP or SWP in milk with that of water would be somewhat spurious. The addition of AWP or FWP to milk caused the freezing point to become abnormally low; if this effect would present a barrier to the acceptance of the use of AWP or FWP for protein 'down' standardization, its magnitude would be overshadowed by the adverse effect of these UF permeates on the heat stability and sensory quality of milk.

As a deviation from the general focus of this work, it was shown that protein standardization of UHT milk, with SMP or AWP, did not obscurate the use of furosine, an indirect product of the early stages of the Maillard reaction, as an indicator of direct or indirect UHT heating. After 1 week storage at 4°C, direct UHT milk products had furosine concentrations in the range 8.5 - 11.5 mg L^{-1}, compared to values of 17.5 - 46 mg L^{-1} for indirect UHT milk samples; substantial fluctuations in the concentration of reactants (lactose and protein) involved in the formation of furosine, caused by protein standardization, did affect the level of furosine in UHT milk, but the effect was insufficient to confound the distinction between direct and indirect UHT milk by measurement of the concentration of furosine. Of greater importance was the impact of temperature of storage of the UHT milk on the level of furosine; at 4°C almost no change in the concentration of furosine occurred, in contrast to substantial increases in furosine concentration when UHT milk was stored at 25°C. If storage conditions were unknown, the use of furosine to distinguish between direct or indirect UHT milk could be compromized.

2 Protein adjustment

- Adjustment of the protein fraction of skim milk, by enrichment with semi-purified fractions of either α-lactalbumin (α-la) or β-lactoglobulin (β-lg) led to markedly different heat stability profiles; in general, the presence of extra α-la in skim milk had a relatively benign effect on heat stability, especially at the unadjusted pH of milk, whereas enrichment of skim milk with β-lg caused a drastic reduction in heat stability, evident over the initial pH range 6.4 - 7.1.

In model solutions, β-lg was shown to be sensitive, whereas α-la was extremely resistant, to heat aggregation. Despite the fact that α-la is known to denature at a lower temperature than β-lg, it can be argued that α-la exhibits a greater overall heat stability,

because of the observed greater resistance of α-la to heat aggregation, when both proteins were subjected to prolonged heating. The contrasting heat stabilities of α-la and β-lg were further exemplified when the protein content of skim milk was adjusted by blending with solutions of α-fraction or β-fraction. When the mass ratio of skim milk to α-fraction solution was in the range 95/5 - 50/50, the heat stability, measured as the heat coagulation time at 140°C, increased slightly near the natural pH of milk (~ 6.6 - 6.7), but was reduced at pH > 6.7. Compared to α-la, the use of β-lg for protein adjustment caused a much larger decline in heat stability; in general, blending of skim milk and β-fraction solution or whey protein concentrate solution, at ratios of 95/5 - 60/40, caused heat stability, measured from pH 6.4 - 7.1, to be reduced greatly, except for a pronounced increase in heat stability at pH 6.8 and a 70/30 ratio of skim milk to β-fraction or whey protein concentrate solution.

More drastic alteration of the protein fraction of milk, though not consistent with the general notion of protein standardization, as interpreted by the International Dairy Federation, could nevertheless present interesting new opportunities for the dairy industry. Whey proteins have a higher proportion of essential amino acids, their biological values exceeding that of even egg white (Sienkiewicz and Riedel, 1990). Moreover, the recent work of McIntosh *et al.* (1995) and Wong and Watson (1995) have demonstrated that whey proteins possess anticarcinogenic properties, being capable of restricting the growth of tumours in laboratory animals. This points to the existence of a marketing opportunity for foods containing whey proteins, which has probably not been exploited fully. It seems possible that fluid milk fortified with a whey protein concentrate or a whey protein fraction could be sold on the basis of an increased nutritional value. However, the results of Chapter VIII indicate that caution would be required in the heat treatment of protein-adjusted milk, where adjustment involved the addition of β-lg-rich products to milk, in contrast to the use of a whey protein fraction of α-la which, at the natural pH of milk, did not adversely affect the heat stability. In view of the high heat stability of milk containing added α-la, and hence its high processability, it would be interesting to establish whether the alleged anticarcinogenic activity of whey is caused by the presence of β-lg, α-la, both of these proteins or other constituents of whey.

Adjustment of the ratio of whey protein to casein protein in milk could also lead to technological benefits. The reduced heat stability of milk containing added β-lg-rich protein

preparations would certainly restrict the ability of such milk to tolerate normal industrial heat processes, but at the same time it is conceivable that lower heat stability could be exploited to manufacture novel dairy products. This is substantiated by the work of de Wit (1989) who demonstrated that heat treatment of skim milk with added whey protein concentrate could be used to form texturized dairy products; however, such products appear to have not received much attention from a commercial viewpoint. Rattray (1992) and Rattray and Jelen (1995) showed that acidification or especially acidification followed by mild heat treatment of a whey protein concentrate solution led to the development of a viscous, custard-like texture with potential application as a fat replacer. In light of their work, it would be interesting to see whether the texture of protein-adjusted skim milk could be favourably altered by judicious heat treatment and/or adjustment of pH.

3 Measurement of freezing point as a tool to detect changes in milk salt equilibria

- Measuring the freezing point (FP) of milk is a simple, yet effective, method of detecting irreversible changes in milk salt equilibria; the irreversibility of the acid dissolution of the colloidal calcium phosphate of milk and the inability of added salts to bind to the milk proteins were demonstrated unambiguously by this technique.

In Chapters IV and VII, it was shown that the presence of small amounts of UF permeate in milk, for the purpose of protein 'down' standardization, could be detected readily by measurement of FP; the presence of SMP or SWP in milk, caused the concentration of soluble salts to be reduced, and hence the FP to be increased, while the addition of AWP or FWP to milk had the opposite effects. The sensitivity of the FP of milk to changes in salt equilibria was confirmed in Chapter IX, where it was shown that acidification of milk led to a reduced FP, due to the dissolution of the colloidal calcium phosphate (CCP) of the casein micelles; at its natural pH, the FP of milk was - 0.522°C, while at pH 4.8, the FP was about - 0.66°C. When acidified milk was neutralized back to its initial pH (~ 6.7) or greater (up to pH 10.0), the FP remained low and approximately constant, indicating that the dissolution of colloidal minerals caused by acidification was a completely irreversible process. Alkalization of milk, from pH 6.7 to pH 10.0, appeared to have no effect on the milk salt equilibria, as the FP was not altered appreciably.

Heating of milk under conditions as harsh as 120°C for 20 min, followed by cooling at 4°C for 20 h, did not produce any change in the response of FP to acidification or acidification followed by neutralization, indicating that changes in milk salt equilibria caused by heating were reversible. It appeared that only very severe heating of milk, namely to its coagulation point could produce a permanent shift in milk salt equilibria; when milk samples, adjusted to the pH range 6.4 - 7.1, were heated, at 130°C for ~ 15 - 40 min, to induce coagulation, FP measurements indicated that a permanent shift from soluble to colloidal salt occurred.

The addition of NaCl, LiCl, $CaCl_2$ or Na_2SO_4 to milk reduced the solubility of the caseins such that, at high levels of added salt they were completely insoluble or were salted out of solution. This phenomenon was not caused by the binding of the added salts to the caseins, because the FP of milk declined in a manner which was exactly predicted for the existence of the added salts in the free solution. The results were not inconsistent with the 'preferential hydration' theory of protein salting-out, which postulates that the addition of cosolute to a solution of protein increases the water 'shell' of protein hydration, thereby providing an entropic driving force for protein association.

The fact that the acid dissolution of CCP was shown to be an irreversible process provokes a number of questions. Firstly, it would be interesting to known how acidification followed by neutralization would affect the structure of the casein micelle. It is known that the acid-induced loss of CCP from the casein micelles is accompanied by an initial dissociation of the caseins, especially β- and κ-casein, from the micelle, followed by aggregation of the dissociated caseins at the isoelectric point (Mulvihill and Grufferty, 1995). Neutralization of acidified milk would be unlikely to restore the native structure of casein micelles, in view of the permanent loss of CCP. The question also arises as to what is the structure and stability of the solubilized salts upon acidification and neutralization of milk; the issue of stability could be investigated by measuring the FP of acidified and neutralized milk over time to determine whether the solubilized CCP would remain soluble or tend to precipitate. The heat stability of acidified and subsequently neutralized milk has not been investigated; such an investigation could shed light on the contribution of casein micelle structure, especially the CCP component, on the heat stability of milk. The inability of alkalization of milk to alter the salt equilibria suggests that acidification of alkalized milk back to the normal pH (~ 6.7) would not alter the structure of casein micelles; possibly,

heat stability would not be altered by an alkalization and acidification treatment, though this appears not to have been confirmed experimentally.

Other, apparently unexplored areas, where the use of FP measurement could prove to be a valuable tool would be assessing the impact of pH adjustment on milk containing added salts; how would the dissolution of CCP by acidification, or the inability of dissolved CCP to reassociate with casein, be affected if the ionic strength of milk was artificially high? Similarly, it is not known how protein standardization would interact with the effect of pH adjustment on the salt equilibria of milk; different responses would be expected for SMP versus AWP, considering that the addition of SMP to milk reduced the concentration soluble salts, while AWP had the opposite effect.

Closing remarks

In conclusion, it can be stated that alteration of the protein fraction of bovine milk, be it minor - protein standardization - or more drastic - protein adjustment, presents ample new opportunities for the dairy industry, but the effect of such modifications on the properties of milk need to be evaluated carefully - in particular, the issues of heat stability, nutritional and sensory quality. A better insight into the protein component of milk can be acquired be investigating its response to heat and its interaction with other milk components and for these purposes measurement of FP appears to be a useful technique.

Finally, let it be said that there is nothing iniquitous in basic science for the sake of curiosity, oblivious to pragmatic and especially economic implications of what may be discovered; while some of the results obtained by this author may be of practical importance, other areas investigated are of a more basic nature, the technological implications of which though uncertain, cannot be ruled out.

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